Remarks

Claims 12-17 and 38-52 were previously pending in this application. Claim 12 has been amended. Support for this amendment can be found in the specification at least on page 35 lines 20-22 and page 41 lines 31-33. No claims have been cancelled. Claims 12-17 and 38-52 are now pending with claim 12 being an independent claim. Claims 12-17, 38-40, 42, 44, 45, 47, 48 and 52 are currently under review, with the remaining pending claims being presently withdrawn as drawn to non-elected species.

No new matter has been added.

Restriction Requirement

Claims 41, 43, 46 and 49-51 are currently withdrawn based on a species election. Upon allowability of the generic claim (i.e., claim 12), examination of the withdrawn claims is requested.

Rejection under 35 U.S.C. §112, second paragraph

Claims 12-17, 38-40, 42, 44, 45, 47, 48 and 52 are rejected under 35 U.S.C. § 112, second paragraph, as being indefinite. The basis of the rejection is unclear. Nevertheless Applicant has amended claim 12 to recite "treating or preventing an asthmatic event" in "hypo-responsive subjects having allergic asthma". Reconsideration and withdrawal of the rejection is respectfully requested.

Rejection under 35 U.S.C. §112, first paragraph, written description

Claims 12-17, 38-40, 42, 44, 45, 47, 48 and 52 are rejected under 35 U.S.C. § 112, first paragraph, as failing to comply with the written description requirement. The Examiner states that the term "allergic asthmatic event" does not have support in the specification. Claim 12 has been amended to recite "asthmatic event" in reference to an event that is experienced by a subject. The claim also recites that the subject to be treated is a hypo-responsive subject having allergic asthma. Support for asthmatic event can be found throughout the specification including but not limited to page 41 lines 31-33. Reconsideration and withdrawal of the rejection is respectfully requested.

Rejection under 35 U.S.C. §112, first paragraph, enablement

Claims 12-17, 38-40, 42, 44, 45, 47, 48 and 52 are rejected under 35 U.S.C. § 112, first paragraph, as failing to comply with the enablement requirement.

Applicant has previously presented a full Wands analysis relating to the enablement of the pending claims. Applicant will not re-iterate that analysis, but will instead rebut the issues raised by the Examiner in the instant Office Action.

The Examiner bases her rejection on unpredictability in the art, an insufficient disclosure in the specification, and a lack of working examples particularly in human subjects. The Examiner attributes unpredictability in the art to a lack of understanding of the mechanism by which CpG immunostimulatory nucleic acids function and uncertainty regarding the safety of the claimed method. Applicants respectfully traverse.

Respectfully the mechanism by which an invention works, the safety of the claimed method, and human clinical trial data are not requirements for enablement of a claimed invention. Neither the Patent Office nor the courts require an understanding of the mechanism underlying a claimed invention. Newman v. Quigg, 877 F.2d 1575, 1581 (Fed. Cir. 1989). Notwithstanding this, it is known that CpG nucleic acids function by binding to Toll-like receptor (TLR) 9 within cells, and that the signaling cascade resulting from this receptor binding leads to induction of a Th1 response and suppression of a Th2 response, effects which are beneficial to the treatment of Th2-mediated disorders such as allergy and asthma.

Similarly the Patent Office and the courts have never required a showing of safety relating to a claimed method of treatment. (See MPEP 2164.01(c) which states "The applicant need not demonstrate that the invention is completely safe.") The determination of whether a treatment method is safe in humans is within the purview of the FDA, not the Patent Office. In re Brana, 51 F.3d 1560 (Fed. Cir. 1995). This determination can only be made via lengthy and costly human clinical trials that are far more likely to be conducted if patent rights relating to the methods are, or are being, secured. Notwithstanding this, Applicant previously cited several phase I and II studies using CpG nucleic acids. These studies demonstrate that CpG nucleic acids are well tolerated in human subjects. (Creticos et al. J Allergy Clin. Immunol. 109(4), 742-743. 2002; Simons et al. J Allergy Clin Immunol 113, 1144-1151 (2004); Krieg et al. J Immunother. 27, 460-471 (2004);

Cooper et al. J Clin. Immunol 24, 693-702 (2004); Halperin et al. Vaccine 21, 2461-2467 (2003); Siegrist et al. Vaccine 23, 615-622 (2004); Cooper et al. Vaccine 22, 3136-3143 (2004); Speiser et al. J Clin. Invest 115, 739-746 (2005); van Ojik et al. Ann. Oncol. 13, 157. 2003. All of these references are submitted herewith for the Examiner's consideration.) These studies should therefore rebut the Examiner's concern that CpG administration in human subjects would be unsafe.

Should the Examiner take issue with the post-filing nature of these references, Applicant stresses that these references are as valid and must be given as much weight as any post-filing reference cited by the Examiner to support her position of unpredictability in the art. The Examiner cannot pick and choose which references are relevant to the issue of predictability in the art. Rather, she must consider these references in their totality, and in doing so consider what one of ordinary skill in the art would take away from these references as a whole.

Furthermore Applicant is not relying on these references to enable the claimed method, but rather to show that CpG administration in humans is safe. They are intended to rebut the Examiner's concern regarding safety. As stated above, the nature of human clinical trials virtually prevents an applicant from having such data prior to beginning the patent process and thus the only reports of such trial data are almost always available post-filing.

To be clear, Applicant continues to maintain that the references cited by the Examiner, whether pre- or post-filing, do not support her position that the art is unpredictable. Applicant refers the Examiner to the prior response which dealt with each of the cited references in detail. In particular, the Examiner's attention is drawn to Krieg et al. 2000 Immunol Today 21:521-526 which teaches that "CpG DNA is effective in asthma immunotherapy even when given as a stand-alone without allergen ..." Moreover, when taken together with the references provided herewith, such references can reasonably be read as predicting the utility of CpG nucleic acids in immune induction in humans.

The Examiner further considers that the disclosure provided by the specification is insufficient since practice of the invention "would require de novo determination of accessible target sites, modes of delivery and formulations of CpG to target appropriate cells and/or tissues". The claimed method relates to the treatment of subjects having allergic asthma which is a form of asthma. Asthma is a condition that affects the airways, and thus the target site, mode of delivery

and CpG formulation would be similar if not identical to other uses of CpG for airway related conditions. There is therefore no requirement for "de novo determination" of these elements, since the condition is known, and products and methods for treating the condition have been previously contemplated (see for example the asthma/allergy medicaments listed in the specification on pages 48-57). Most if not all of these products are administered to the airways. The Examiner acknowledges that the specification teaches airway administration (see page 11 of the Office Action), however she challenges such teachings as being prophetic. The Patent Office allows prophetic examples, as the Examiner should be well aware. In addition, whether prophetic or not, one of ordinary skill would not question the use of airway administration and related formulations for the treatment of an airway-mediated condition. The amount of disclosure provided by the specification is more than sufficient, particularly in view of the high level of skill in the art, as stated in the prior response.

Finally, Applicant has amended claim 12 to recite nucleic acids that are 8-100 nucleotides in length. Accordingly, the Examiner's reliance on McCluskie et al. Mol Med. 1999 5:287-300 is no longer applicable since the reference relates to DNA vaccination which uses nucleic acids such as plasmids or nucleic acids vectors which encode antigens.

The Wands analysis of the claimed invention therefore amounts to a lack of working examples. Applicant however reiterates that a lack of working examples alone is not dispositive of enablement. This is especially so where, as in this case, the Examiner appears to require nothing less than human clinical trial data to establish safety and utility. This is an unreasonable burden to place on any Applicant, and it is not supported by the case law.

In summary, Applicant maintains that the art of CpG immunotherapy, including CpG immunotherapy in the context of airway disorders, was predictable at the time of filing, and that the specification provided sufficient guidance for one of ordinary skill in the art to practice the invention. The Examiner is referred to the previous filed response for a discussion of the other Wands factors, all of which when taken in their totality establish enablement of the claimed invention at the time of filing.

Reconsideration and withdrawal of the rejection under 35 U.S.C. §112 is respectfully requested.

Conclusion

A Notice of Allowance is respectfully requested. If this response is not considered timely filed and if a request for an extension of time is otherwise absent, Applicant hereby requests any necessary extension of time. If there is a fee occasioned by this response, including an extension fee, that is not covered by an enclosed check, please charge any deficiency to Deposit Account No. 23/2825.

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Respectfully submitted,

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New approaches in immunotherapy: allergen vaccination with immunostimulatory DNA

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Allergen immunotherapy, first introduced in the early part of the twentieth century, remains a cornerstone of the practicing allergists' approach to the treatment of allergic respiratory diseases. It is comprised of a time-honored approach that aims to modulate the immune response and attenuate or eliminate symptoms through administration of increasing doses of an extract that is comprised of the aeroallergens to which the patient is allergic. Controlled clinical trials have demonstrated therapeutic efficacy and detailed the favorable immunologic changes associated with allergen immunotherapy for allergic rhinitis, asthma, and venom sensitivity [1–7].

Despite its success, conventional immunotherapy is saddled with a number of encumbrances. Impediments include the need for frequent dosing over years, which impacts patient compliance; the need to administer a relatively large dose of the immunizing agent to achieve control of symptoms; and the potential for clinically significant allergic reactions to the treatment. Considerable effort has been devoted to developing improved therapeutic vaccines for allergic diseases to: (1) improve efficacy, (2) decrease the time required to achieve effect, (3) reduce inconvenience and improve compliance with immunization regimens, and (4) enhance safety.

Recognizing that allergen dose is limited by the potential for systemic reactions to the respective allergens, efforts largely have been directed at decreasing

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the allergenicity (ie, the potential for inducing an allergic reaction) of the immunizing antigens while preserving their immunogenicity (ie, the ability to induce a beneficial immunologic response). Although various chemical modifications of allergens have been attempted over the past several decades, the end result has been that allergenicity and immunogenicity decreased or increased in tandem, resulting in no improved risk–benefit ratio.

An allergenic vaccine that could reduce allergenicity, maintain immunogenicity, and be given in a few doses would have important therapeutic implications, as millions of patients with poorly controlled allergic rhinitis and asthma would be candidates for such a form of immunomodulation.

This article addresses a specific adjuvant approach to immunotherapy in which highly active immunostimulatory phosphorothioate oligodeoxyribonucleotide (ISS-ODN) moieties are linked to the principal allergenic moiety of a relevant aeroallergen (eg, ragweed Amb a 1) [8,9]. This adjuvant approach may prove to be highly effective at directing the immune response toward a more favorable T helper cell type 1 (Th1) phenotypic expression to counterbalance the untoward Th2-driven proinflammatory allergic process.

The allergic diathesis

To develop improved materials for immunotherapy, it is important to recognize the pathophysiology that underpins the allergic diathesis. On allergen exposure, susceptible individuals develop acute allergic responses that are indicative of IgE-dependent mast cell activation, which results in the release of preformed chemicals (histamine, various enzymes), newly generated inflammatory mediators of the arachidonic acid pathway (leukotrienes, prostaglandins), and cytokine molecules [10–12]. Tangential to this IgE-mediated allergic reaction, allergen also is processed by antigen-presenting cells that are displayed in association with class II human leukocyte antigen molecules and presented to T-lymphocytes. If appropriate, co-stimulatory signals are induced, resulting in T-cell activation, induction of Th2 cell development, recruitment to the site of allergic inflammation, and subsequent elaboration of proinflammatory cytokines (eg, interleukin 4 [IL-4], IL-5, IL-13) [13–16]. These cytokines help to amplify and sustain the overall response by regulating IgE synthesis, eosinophil survival, eosinophil migration, and mucus production, resulting in airway inflammation. The identification of transcription factors controlling Th1 and Th2 development further support this overall hypothesis, in that GATA3 (Th2-associated) is overexpressed in the asthmatic airway, whereas T-bet (Th1-associated) is underexpressed [17,18].

Mechanism of conventional immunotherapy

The authors' allergy group at Johns Hopkins took an early lead in examining the mechanisms by which allergen immunotherapy affects clinical improvement

in patients with allergic disease. These studies demonstrated that immunization initially resulted in production of IgG and IgE antibodies by antigen-specific B cells. With continuing immunotherapy, IgG levels further increased and plateaued, whereas antigen-specific IgE titers gradually declined toward pretreatment levels and were not boosted by subsequent environmental exposure to the allergen [19–22]. In conventional immunotherapy, induction of IgG antibodies is a predictor of clinical success, albeit clinical benefit is not likely to be achieved until the immunizing doses are large enough to risk anaphylaxis.

Various challenge models have been used as tools with which to evaluate the effects of immunotherapy. Specific nasal allergen provocation and nasal biopsy studies of patients with pollen sensitivity have been used to demonstrate that immunotherapy shifts the Th2–Th1 paradigm. Patients who successfully were immunized demonstrated attenuation of the acute challenge response (less inflammatory mediator release in nasal secretions) and an abrogation of the late-phase allergic reaction (decreased eosinophil and basophil migration and mediator release) [23–26]. Nasal biopsies of patients with rhinitis who were immunized against grass pollen and subsequently underwent challenge with grass pollen extract showed a significant increase in mRNA for specific Th1 cytokines (interferon γ [IFN-γ], IL-12) and a significant reduction in allergen-induced accumulation of total numbers of $CD4^+$ T cells and eosinophils in the nasal mucosa. These techniques have resulted in little down-regulation in the expression of cytokines that are hallmarks of Th2 responses (eg, IL-4, IL-5) [27–30].

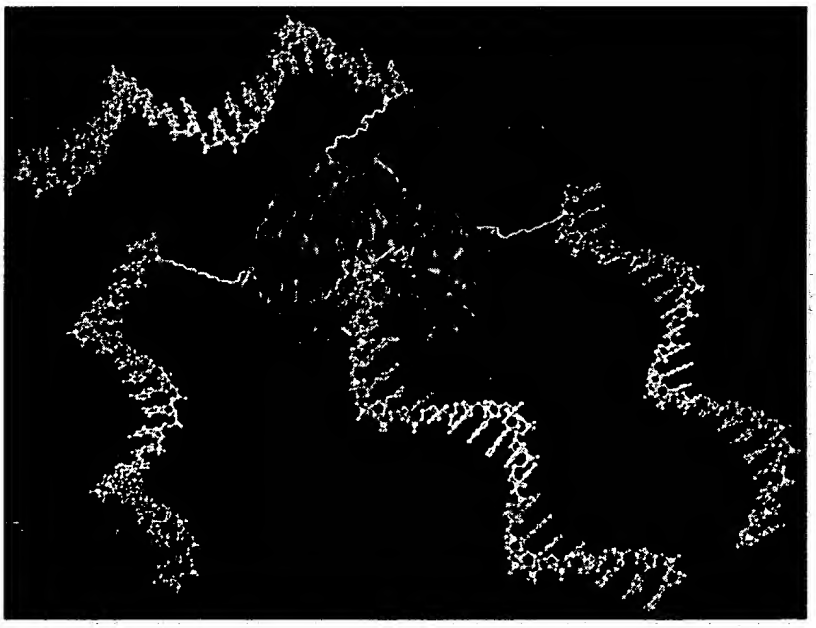

Fig. 1. AIC showing four molecules of ISS linked to purified ragweed Amb a 1.

Tangential to these observations, Secrist et al [31] cultured peripheral blood mononuclear cells from allergic patients receiving maintenance grass-pollen immunotherapy and demonstrated a significant decrease in allergen-induced IL-4 synthesis when these cells were exposed to allergen in vitro.

These collective studies demonstrate that immunotherapy has the potential to down-regulate the immediate-phase allergic reaction and T-cell–mediated late-phase responses; however, allergen dose is limited by the potential for severe systemic reactions in sensitized patients. At issue is the ability to administer a dose that is large enough to induce the desired therapeutic changes without causing intolerable allergic side effects. Raz et al [8] developed a novel immunostimulatory DNA construct termed AIC (Dynavax Technologies). This experimental vaccine is comprised of specific immunostimulatory oligdeoxyribonucleotide sequences (ISSs) that are conjugated to the principal allergenic moiety of ragweed (Amb a 1; Fig. 1). AIC has been shown to induce an enhanced, ragweed-specific, Th1-type response in mice, compared with Amb a 1 alone or Amb a 1 plus alum [32].

Background for adjuvant approaches

Various molecules, including lipopolysaccharides, aluminum hydroxide salts, and Freund's adjuvant, have been shown to possess immunostimulatory properties that can enhance the response to antigen. Various research laboratories have attempted to capitalize on this observation to improve a vaccine's immunogenicity through an enhanced adjuvant effect. In their study of the active components of Freund's adjuvant, Tokunaga et al [33–35] made the initial discovery of the adjuvant effect of bacterial DNA. Krieg et al [36] identified unmethylated CG dinucleotides, termed CpG motifs, as the moieties responsible for the immunostimulatory effect of bacterial DNA. These immunostimulatory properties are unique to certain bacterial DNAs and are absent in vertebrate DNA. This uniqueness is caused by the higher frequency of unmethylated CpG motifs in bacterial DNA as contrasted to vertebrate DNA. Without these differences, the immunostimulatory activity would be abolished [34,37,38].

The effects of bacterial DNAs can be mimicked using synthetic oligodeoxyribonucleotides (ODN), allowing a more accurate definition of the bacterial DNA immunostimulatory sequences (Box 1). Early research studies identified optimal ISSs containing palindromic hexamers based on the general formula of 5′-purine-purine-CG-pyrimidine-pyrimidine-3′ (eg, 5′-GACGTC-3′, 5′-AGCGCT-3′, 5′-AACGTT-3′) [36]. More recent work has extended this finding to the identification of different classes of CpG ODN based on their ability to preferentially activate plasmacytoid dendritic cells (PDCs), natural killer (NK) cells, or B cells. CpG-A ODN contains phosphodiester backbones and is effective at inducing interferon α production from PDCs and activating NK cells. CpG-B ODN possesses phosphorothioate backbones that enhance B-cell proliferation and immunoglobulin production, but is less capable of activating PDCs and NK

Box 1. Synthetic oligodeoxyribonucleotide immunostimulatory sequences

- Believed to be taken up by professional antigen-presenting cells (macrophages, dendritic cells)
- Resulting in secretion of specific co-stimulatory factors (IL-12, IL-18, TNF-α, IFN-α, IFN-β)
- Activating a variety of immune effector cells (T cells, NK cells) and driving the adaptive immune response toward a Th1 phenotype
- Inducing a potent humoral adjuvant effect
- Enhanced immunostimulatory activity observed if ISS is coupled to allergen (AIC)

cells [39,40]. CpG-C represents a new class of ODN, possesses characteristics of the A and B classes, and is capable of activating B cells and PDCs [41,42].

Immunostimulatory oligodeoxyribonucleotide sequences induce Th1-type response and inhibit Th2-type immune response

Research has shown that CpG acts through toll-like receptor (TLR) 9 [43]. The association of CpG with TLR9 occurs intracellularly within lysomes [44]; this interaction induces signals through the myeloid differentiation factor 88 pathway, eventually activating nuclear factor κB (NF-κB), much like what has been described for other TLR1 ligand interactions [45]. TLR9 was found to be expressed predominantly on human PDCs, basophils, and B cells, although some cell types have yet to be tested [46,47]. CpG-A ODN strongly stimulates PDCs to produce high amounts of IFN-α and IL-12, which activate IFN-γ–producing NK cells and γδ T cells that drive immune cells toward Th1 responses (Fig. 2) [40,48–51].

Low concentrations of CpG ODN lead to an increase in B-cell proliferation, antigen-specific immunoglobulin secretion, and IL-6 production [36,52]. Exposure of tonsilar B cells to CpG ODN induces a concentration- and time-dependent up-regulation of the activation markers CD23, CD25, CD40, CD54, CD80, CD86, and HLA-DR, and counteracts IgE production induced by IL-4 [53]. CpG directly induces T-bet expression and inhibits IgG1 and IgE switching in B cells, which is induced by IL-4 and CD40 signaling [54]. In murine models, pre-administration of CpG ODN in vivo prevented allergen-induced Th2 responses, including IL-5 production, eosinophilic airway inflammation, airway hyper-reactivity, and chronic airway remodeling [55–59].

CpG, or ISS-ODN, seems to stimulate the innate immune system to produce cytokines that drive the adaptive immune system toward a Th1-type immune

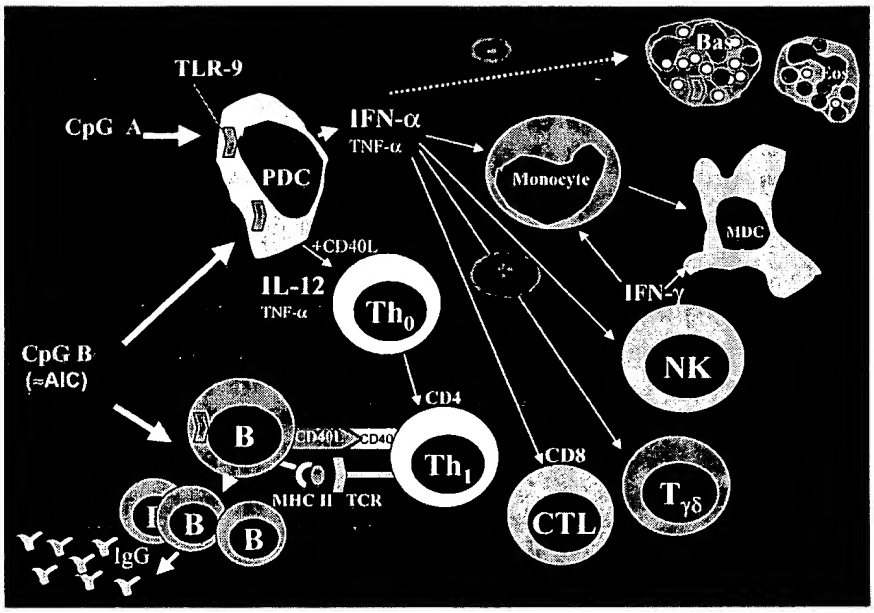

Fig. 2. The effects of CpG ODN on the human immune system. B, B cell; Bas, basophil; CTL, cytotoxic T lymphocyte; Eos, eosinophil; MDC, monocyte-derived dendritic cell; MHC, major histocompatibility complex; NK, natural killer cell; PDC, plasmacytoid dendritic cell; TCR, T cell receptor.

response and inhibit Th2-type responses. This finding suggests a potential clinical use for CpG in the immunotherapy of allergic diseases.

Effects of CpG-based vaccines on immune response

Conjugation of allergen with ISS-ODN (AIC) was developed because it was believed that AIC may be more immunogenic and less allergenic than a simple cocktail of allergen mixed with ISS-ODN [8]. The immunomodulatory effect of direct linkage versus conjugation of allergen with ISS is demonstrated in Fig. 3. This graph shows that in a murine animal model, immunization with a conjugate of Amb a 1 and 1018 ISS (AIC) promoted IgG2a antibody production that is significantly greater than that observed with allergen mixed with ISS, allergen plus alum, or allergen alone. An inhibition of IgE production has been a corollary finding to this protective IgG antibody response. These observations are particularly relevant, considering that the murine-model IgG2a is a marker of induction of a Th1 response, whereas IgE production is induced through Th2 mechanisms. These reciprocal IgG and IgE antibody responses are similarly reproducible in previously sensitized and not simply naive animals [8,32]. This factor offers the opportunity to use this approach as prophylactic intervention and a therapeutic construct in sensitized individuals who are in the midst of an allergic diathesis.

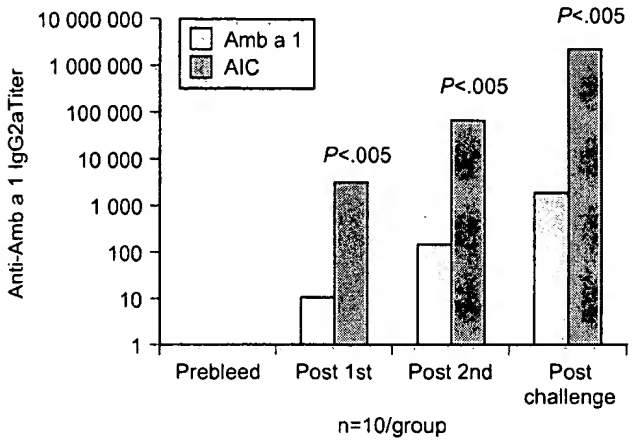

Fig. 3. IgG2a anti-Amb a 1 response in mice. Effect of immunization with AIC (*solid bar*) versus Amb a 1 alone (*shaded bar*) on IgG2a antibody production. Assays obtained pre-immunization, post-immunization (after the first and second vaccinations), and after challenge. Not shown are allergen mixed with ISS or allergen plus alum. (*Adapted from* Creticos PS. New methods in asthma and allergic rhinitis. Advanced Studies in Medicine 2002;2:273; with permission.)

AIC use results in the preferential induction of naïve $CD4^+$ T cells, resulting in the differentiation toward a Th1 phenotypic profile. The corollary to this observation of a down-regulation in Th2-mediated responses to allergen (eg, IL-4, IL-5) is believed to be the result of dendritic cell (DC), macrophage or monocytic activation, and production of cytokines (IL-12, IL-18, IFN-α, IFN-β),

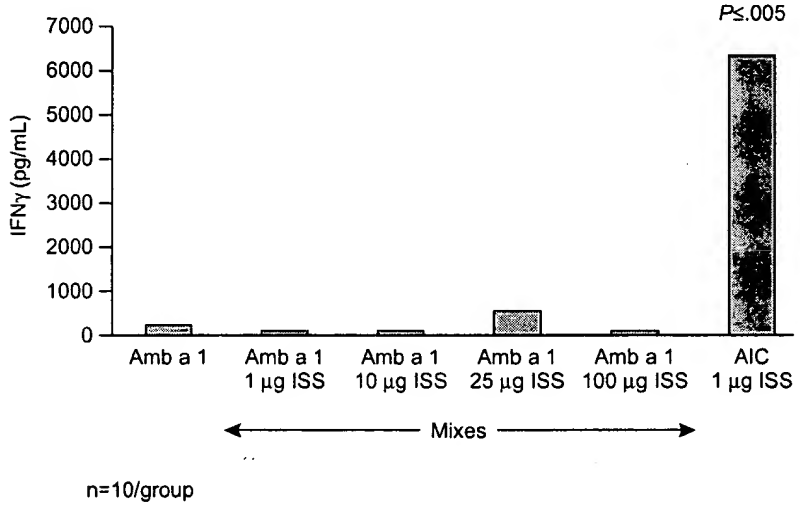

Fig. 4. In mice, effect of immunization on up-regulation of IFN-γ: AIC versus Amb a 1–ISS mixes versus Amb a 1 alone. (*Adapted from* Creticos PS. New methods in asthma and allergic rhinitis. Advanced Studies in Medicine 2002;2:273; with permission.)

with constituent effects on priming for Th1 development that results in increased IFN-γ on activation [8,9,32]. In a murine model, AIC immunization resulted in the specific up-regulation of IFN-γ (Th1 profile), which contrasts the Th2 cytokine dysregulation that otherwise would be observed from immunization with allergen alone, allergen plus alum, or allergen mixed with ISS but not linked to this specific adjuvant (Fig. 4). Marshall et al [9] observed similar results in in vitro studies of peripheral blood mononuclear cells that were obtained from human subjects with ragweed allergy. This research demonstrates that cells exposed to AIC produced significant levels of IFN-γ and had diminished production of IL-4 and IL-5.

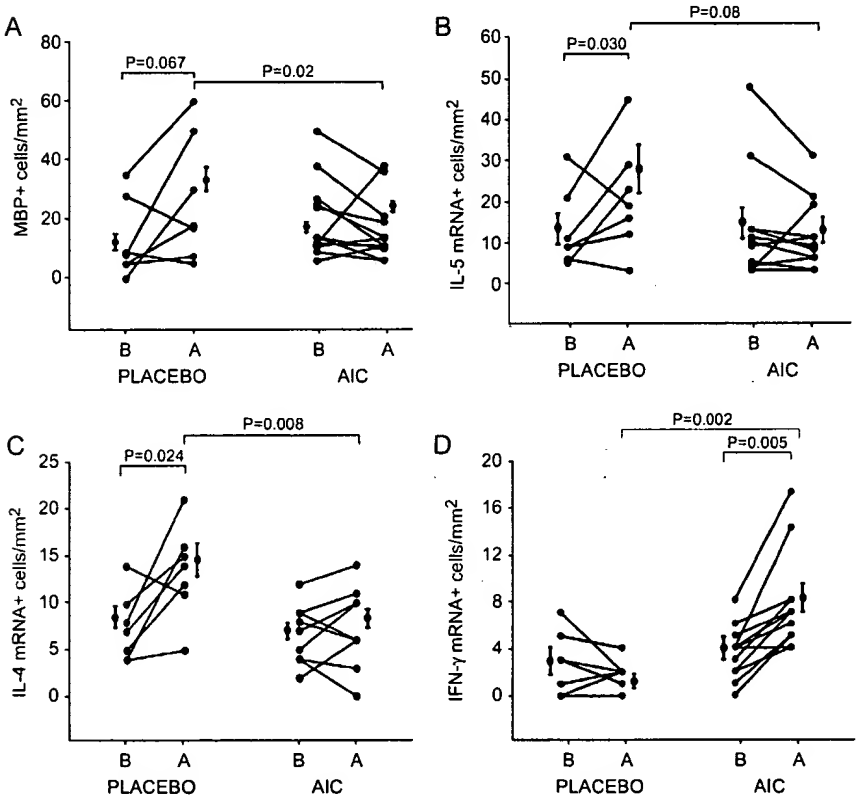

Fig. 5. Effect of AIC on allergen challenge before the ragweed season. The number of major basic protein (MBP)-positive cells (*A*), IL-5 mRNA-positive cells (*B*), IL-4 mRNA-positive cells (*C*), and IFN-γ mRNA-positive cells (*D*) at baseline (B) and after nasal allergen challenge with ragweed extract (A) in ragweed-sensitive patients 2 weeks after treatment with placebo (n = 7) or AIC (n = 12) is shown. Circles alongside the data represent mean values ± SEM. (*Adapted from* Tulic MK, Fiset PO, Christodoulopoulos P, Vaillancourt P, Desrosiers M, Lavigne F, et al. Amb a 1-immunostimulatory oligodeoxynucleotide conjugate immunotherapy decreases the nasal inflammatory response. J Allergy Clin Immunol 2004;113:239; with permission.)

Horner et al [60] showed that an optimal conjugation ratio of ISS-to-Amb a 1 resulted in an AIC that was approximately 100-fold less allergenic than native allergen, as measured by mast cell degranulation. Use of AIC also resulted in a marked reduction in anaphylactic death, and similar results were seen in a murine model of the Arthus reaction.

Clinical trials with Amb a 1 immunostimulatory oligonucleotide construct

Various clinical trials have been undertaken with the experimental AIC construct in France, Canada, and the United States. Data from the European studies have confirmed the safety of AIC when patients were immunized to 30 μg of Amb a 1 equivalent product [61]. The Canadian study by Hamid et al [62] demonstrated that AIC treatment resulted in a decrease in eosinophil migration and a shift from a Th2 profile to a Th1 profile (Fig. 5).

The authors' group at Johns Hopkins completed a Food and Drug Administration-defined clinical safety study in which quantitative intradermal endpoint skin titration was employed to assess the relative potency of AIC to a standardized, commercially available ragweed preparation. The results demonstrated that the AIC product was more than 100-fold less reactive than the licensed ragweed extract when assessed with this parallel-line bioassay methodology. In a subset of patients, in vitro studies of basophil histamine release showed a similarly positive effect to that observed with the in vivo skin titration studies [64].

The authors' initial injection study demonstrated the tolerability and immunogenicity of AIC. Patients with ragweed allergy safely were dosed to 15 μg of AIC (based on Amb a 1 content) and demonstrated an immune response that was consistent with that for conventional ragweed immunotherapy [63].

The authors' clinical trial through the Immune Tolerance Network of the National Institutes of Health demonstrated encouraging clinical efficacy in patients with fall-seasonal allergic rhinitis. Patients experienced improvement in visual analog scores, seasonal symptom diary scores, quality-of-life scores, and medication use. These positive clinical observations were observed in the initial ragweed season, and improvement continued during the second season of observation, findings that provide evidence of sustained benefit [65].

Summary

The results from these clinical studies of patients with ragweed-induced allergic rhinitis demonstrated that AIC is less allergenic than conventional immunotherapeutic products and may offer the potential for an improved safety profile for immunotherapy. The initial phase I and phase II clinical trials with AIC demonstrated the improved immunogenicity and therapeutic potential of the construct and suggested that AIC may be a superior therapeutic agent, when compared with conventional immunotherapy. Further trials are underway to

evaluate and confirm the optimal dosing, long-term safety, immunologic effect, and therapeutic efficacy of AIC as a treatment modality for ragweed-induced allergic rhinitis and asthma.

References

[1] Durham SR, Varney V, Gaga M, et al. Immunotherapy and allergic inflammation. Clin Exp Allergy 1991;21(Suppl 1):206–10.
[2] Creticos PS. Immunotherapy with allergens. JAMA 1992;268:2834–9.
[3] Creticos PS. Immunotherapy. In: Rich RR, Shearer WT, editors. Clinical immunology, principles and practice. St. Louis (MO): Mosby; 1995. p. 2002–18.
[4] Creticos PS, Reed CE, Norman PS, et al. Ragweed immunotherapy in adult asthma. N Engl J Med 1996;334:501–6.
[5] Creticos PS. The consideration of immunotherapy in the treatment of allergic asthma. J Allergy Clin Immunol 2000;105:S559–74.
[6] Durham SR, Walker SM, Varga EM, et al. Long-term clinical efficacy of grass-pollen immunotherapy. N Engl J Med 1999;341:468–75.
[7] Bousquet J, Lockey R, Malling HJ, et al. Allergen immunotherapy: therapeutic vaccines for allergic diseases: World Health Organization, American Academy of Allergy, Asthma and Immunology. Ann Allergy Asthma Immunol 1998;81:401–5.
[8] Tighe H, Takabayashi K, Schwartz D, et al. Conjugation of immunostimulatory DNA to the short ragweed allergen amb a 1 enhances its immunogenicity and reduces its allergenicity. J Allergy Clin Immunol 2000;106:124–34.
[9] Marshall JD, Abtahi S, Eiden JJ, et al. Immunostimulatory sequence DNA linked to the Amb a 1 allergen promotes T(H)1 cytokine expression while downregulating T(H)2 cytokine expression in PBMCs from human patients with ragweed allergy. J Allergy Clin Immunol 2001;108:191–7.
[10] Ishizaka T, Hirata F, Ishizaka K, et al. Stimulation of phospholipid methylation. Ca2 + influx, and histamine release by bridging of IgE receptors on rat mast cells. Proc Natl Acad Sci U S A 1980;77:1903–6.
[11] Wasserman Sl. Mediators of immediate ypersensitivit. J Allergy Clin Immunol 1983;372: 101–15.
[12] Lewis RA, Austen KF. Mediation of local homeostasis and inflammation by leukotrienes and other mast cell dependet compounds. Nature 1981;293:103–8.
[13] Larche M, Robinson DS, Kay AB. The role of T lymphocytes in the pathogenesis of asthma. J Allergy Clin Immunol 2003;111:450–63.
[14] O'Hehir RE, Garman RD, Greenstein JL, Lamb JR. The specificity and regulation of T-cell responsiveness to allergens. Annu Rev Immunol 1991;9:67–95.
[15] Robinson DS, Hamid Q, Jacobson M, et al. Evidence for Th2-type T helper cell control of allergic disease in vivo. Springer Semin Immunopathol 1993;15:17–27.
[16] Hamid QA, Schotman E, Jacobson MR, Walker SM, Durham SR. Increases in IL-12 messenger RNA + cells accompany inhibition of allergen-induced late skin responses after successful grass pollen immunotherapy. J Allergy Clin Immunol 1997;99:254–60.
[17] Nakamura Y, Ghaffar O, Olivenstein R, Taha RA, Soussi-Gounni A, Zhang DH, et al. Gene expression of the GATA-3 transcription factor is increased in atopic asthma. J Allergy Clin Immunol 1999;103:215–22.
[18] Finotto S, Neurath MF, Glickman JN, Qin S, Lehr HA, Green FH, et al. Development of spontaneous airway changes consistent with human asthma in mice lacking T-bet. Science 2002;295:336–8.
[19] Creticos PS, Van Metre TE, Mardiney MR, et al. Dose response of IgE and IgG antibodies during ragweed immunotherapy. J Allergy Clin Immunol 1984;73:94–104.
[20] Creticos PS. Immunotherapy with allergens. JAMA 1997;268:2834–9.

[21] Lichtenstein LM, Ishizaka K, Norman PS, Hill BM. IgE antibody measurements in ragweed hay fever: relationship to clinical severity and the results of immunotherapy. J Clin Invest 1973; 52:472–82.
[22] Haugaard L, Dahl R, Jacobsen L. A controlled dose-response study of immunotherapy with standardized, partially purified extract of house dust mite: clinical efficacy and side effects. J Allergy Clin Immunol 1993;91:709–22.
[23] Creticos PS, Adkinson Jr NF, Kagey-Sobotka A, et al. Nasal challenge with ragweed pollen in hay fever patients: effect of immunotherapy. J Clin Invest 1985;76:2247–53.
[24] Creticos PS, Marsh DG, Proud D, et al. Responses to ragweed-pollen nasal challenge before and after immunotherapy. J Allergy Clin Immunol 1989;84:197–205.
[25] Iliopoulos O, Proud D, Adkinson Jr NF, et al. Effects of immunotherapy on the early, late, and rechallenge nasal reaction to provocation with allergen: changes in inflammatory mediators and cells. J Allergy Clin Immunol 1991;87:855–66.
[26] Furin MJ, Norman PS, Creticos PS, et al. Immunotherapy decreases antigen-induced eosinophil cell migration into the nasal cavity. J Allergy Clin Immunol 1991;88:27–32.
[27] Varney VA, Hamid QA, Gaga M, et al. Influence of grass pollen immunotherapy on cellular infiltration and cytokine mRNA expression during allergen-induced late-phase cutaneous responses. J Clin Invest 1993;92:644–51.
[28] Durham SR, Ying S, Varney VA, et al. Grass pollen immunotherapy inhibits allergen-induced infiltration of CD4 + T lymphocytes and eosinophils in the nasal mucosa and increases the number of cells expressing messenger RNA for interferon-gamma. J Allergy Clin Immunol 1996;97: 1356–65.
[29] Ebner C, Siemann U, Bohle B, et al. Immunological changes during specific immunotherapy of grass pollen allergy: reduced lymphoproliferative responses to allergen and shift from TH2 to TH1 in T-cell clones specific for Phl p 1, a major grass pollen allergen. Clin Exp Allergy 1997;27:1007–15.
[30] Durham SR, Till SJ. Immunologic changes associated with allergen immunotherapy. J Allergy Clin Immunol 1998;102:157–64.
[31] Secrist H, Chelen CJ, Wen Y, Marshall JD, Umetsu DT. Allergen immunotherapy decreases interleukin 4 production in CD4 + T cells from allergic individuals. J Exp Med 1993;178: 2123–30.
[32] Tighe H, Takabayashi K, Schwartz D, et al. Conjugation of protein to immunostimulatory DNA results in a rapid, long-lasting and potent induction of cell-mediated and humoral immunity. Eur J Immunol 2000;30:1939–47.
[33] Tokunaga T, Yamamoto H, Shimada S, et al. Antitumor activity of deoxyribonucleic acid fraction from *Mycobacterium bovis* BCG: I. Isolation, physicochemical characterization, and antitumor activity. J Natl Cancer Inst 1984;72:955–62.
[34] Yamamoto S, Yamamoto T, Shimada S, et al. DNA from bacteria, but not from vertebrates, induces interferons, activates natural killer cells and inhibits tumor growth. Microbiol Immunol 1992;36:983–97.
[35] Yamamoto T, Yamamoto S, Kataoka T, Tokunaga T. Ability of oligonucleotides with certain palindromes to induce interferon production and augment natural killer cell activity is associated with their base length. Antisense Res Dev 1994;4:119–22.
[36] Krieg AM, Yi AK, Matson S, et al. CpG motifs in bacterial DNA trigger direct B-cell activation. Nature 1995;374:546–9.
[37] Bird AP. DNA methylation and the frequency of CpG in animal DNA. Nucleic Acids Res 1980; 8:1499–504.
[38] Kuramoto E, Yano O, Kimura Y, et al. Oligonucleotide sequences required for natural killer cell activation. Jpn J Cancer Res 1992;83:1128–31.
[39] Krieg AM. CpG motifs in bacterial DNA and their immune effects. Annu Rev Immunol 2002;20:709–60.
[40] Krug A, Rothenfusser S, Hornung V, et al. Identification of CpG oligonucleotide sequences with high induction of IFN-alpha/beta in plasmacytoid dendritic cells. Eur J Immunol 2001; 31:2154–63.

[41] Duramad O, Fearon KL, Chan JH, Kanzler H, Marshall JD, Coffman RL, et al. IL-10 regulates plasmacytoid dendritic cell response to CpG-containing immunostimulatory sequences. Blood 2003;102:4487–92.
[42] Marshall JD, Hessel EM, Gregorio J, Abbate C, Yee P, Chu M, et al. Novel chimeric immunomodulatory compounds containing short CpG oligodeoxyribonucleotides have differential activities in human cells. Nucleic Acids Res 2003;31:5122–33.
[43] Hemmi H, Takeuchi O, Kawai T, et al. A Toll-like receptor recognizes bacterial DNA. Nature 2000;408:740–5.
[44] Latz E, Schoenemeyer A, Visintin A, Fitzgerald KA, Monks BG, Knetter CF, et al. TLR9 signals after translocating from the ER to CpG DNA in the lysosome. Nat Immunol 2004;5: 190–8.
[45] Sabroe I, Parker LC, Wilson AG, Whyte MKB, Dower SK. Toll-like receptors:their role in allergy and non-allergic inflammatory disease. Clin Exp Allergy 2002;32:984–9.
[46] Hornung V, Rothenfusser S, Britsch S, et al. Quantitative expression of toll-like receptor 1-10 mRNA in cellular subsets of human peripheral blood mononuclear cells and sensitivity to CpG oligodeoxynucleotides. J Immunol 2002;168:4531–7.
[47] Kadowaki N, Ho S, Antonenko S, et al. Subsets of human dendritic cell precursors express different toll-like receptors and respond to different microbial antigens. J Exp Med 2001;194: 863–9.
[48] Rothenfusser S, Hornung V, Krug A, et al. Distinct CpG oligonucleotide sequences activate human gamma delta T cells via interferon-alpha/-beta. Eur J Immunol 2001;31:3525–34.
[49] Bauer M, Redecke V, Ellwart JW, et al. Bacterial CpG-DNA triggers activation and maturation of human CD11c-, CD123 + dendritic cells. J Immunol 2001;166:5000–7.
[50] Rothenfusser S, Tuma E, Endres S, Hartmann G. Plasmacytoid dendritic cells: the key to CpG(1). Hum Immunol 2002;63:1111–9.
[51] Krug A, Towarowski A, Britsch S, et al. Toll-like receptor expression reveals CpG DNA as a unique microbial stimulus for plasmacytoid dendritic cells which synergizes with CD40 ligand to induce high amounts of IL-12. Eur J Immunol 2001;31:3026–37.
[52] Yi AK, Klinman DM, Martin TL, Matson S, Krieg AM. Rapid immune activation by CpG motifs in bacterial DNA: systemic induction of IL-6 transcription through an antioxidant-sensitive pathway. J Immunol 1996;157:5394–402.
[53] Gantner F, Hermann P, Nakashima K, et al. CD40-dependent and -independent activation of human tonsil B cells by CpG oligodeoxynucleotides. Eur J Immunol 2003;33:1576–85.
[54] Liu N, Ohnishi N, Ni L, Akira S, Bacon KB. CpG directly induces T-bet expression and inhibits Ig(gamma1) and Ig(epsilon) switching in B cells. Nat Immunol 2003;4:687–93.
[55] Broide D, Schwarze J, Tighe H, et al. Immunostimulatory DNA sequences inhibit IL-5, eosinophilic inflammation, and airway hyperresponsiveness in mice. J Immunol 1998;161: 7054–62.
[56] Broide DH, Stachnick G, Castaneda D, et al. Systemic administration of immunostimulatory DNA sequences mediates reversible inhibition of Th2 responses in a mouse model of asthma. J Clin Immunol 2001;21:175–82.
[57] Kline JN, Waldschmidt TJ, Businga TR, et al. Modulation of airway inflammation by CpG oligodeoxynucleotides in a murine model of asthma. J Immunol 1998;160:2555–9.
[58] Sur S, Wild JS, Choudhury BK, et al. Long term prevention of allergic lung inflammation in a mouse model of asthma by CpG oligodeoxynucleotides. J Immunol 1999;162:6284–93.
[59] Jain VV, Kitagaki K, Businga T, et al. CpG-oligodeoxynucleotides inhibit airway remodeling in a murine model of chronic asthma. J Allergy Clin Immunol 2002;110:867–72.
[60] Horner AA, Takabayashi K, Beck L, et al. Optimized conjugation ratios lead to allergen immunostimulatory oligodeoxynucleotide conjugates with retained immunogenicity and minimal anaphylactogenicity. J Allergy Clin Immunol 2002;110:413–20.
[61] Dieudonné F, Vital Durand D, Eiden J, Tuck G, Van Nest G, Raz E, Hamilton R, et al. ISS linked to Amb a 1 allergen (AIC) stimulates IgG response to Amb a 1 by ragweed-allergic humans [abstract]. J Allergy Clin Immunol 2001;107:933.

[62] Tulic MK, Fiset PO, Christodoulopoulos P, Vaillancourt P, Desrosiers M, Lavigne F, et al. Amb a 1-immunostimulatory oligodeoxynucleotide conjugate immunotherapy decreases the nasal inflammatory response. J Allergy Clin Immunol 2004;113:235–41.

[63] Creticos PS, Eiden JJ, Balcer SL, Van Nest G, Kagey-Sobotka A, Tuck SF, et al. Immunostimulatory oligonucleotides conjugated to Amb a 1: safety, skin test reactivity, and basophil histamine release [abstract]. J Allergy Clin Immunol 2000;105:215.

[64] Creticos PS, Balcer SL, Schroeder JT, Hamilton RG, Chung B, Norman P, et al. Initial immunotherapy trial to explore the safety, tolerability and immunogenicity of subcutaneous injections of an Amb a 1 immunostimulatory oligonucleotide conjugate [AAIC] in ragweed allergic adults [abstract]. J Allergy Clin Immunol 2001;107(Part 2):S216.

[65] Creticos PS, Eiden JJ, Broide DH, Balcer-Whaley SL, Schroeder JT, Khattignavong A, et al. Immunotherapy with immunostimulatory oligonucleotides linked to purified ragweed Amb a 1 allergen: effects on antibody production, nasal allergen provocation, and ragweed seasonal rhinitis [abstract]. J Allergy Clin Immunol 2002;109:743–4.

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CPG 7909, an Immunostimulatory TLR9 Agonist Oligodeoxynucleotide, as Adjuvant to Engerix-B® HBV Vaccine in Healthy Adults: A Double-Blind Phase I/II Study

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Oligodeoxynucleotides containing immunostimulatory CpG motifs (CpG ODN) act as potent Th1-like immune enhancers with many antigens in animal models. We have extended these observations to the first clinical evaluation of the safety, tolerability and immunogenicity of CPG 7909 when added to a commercial HBV vaccine. In a randomized, double-blind phase I dose escalation study, healthy volunteers aged 18–35 years were vaccinated at 0, 4 and 24 weeks by intramuscular injection with Engerix-B® (GlaxoSmithKline). The regular adult dose of 20 μg recombinant hepatitis B surface antigen (HBsAg) adsorbed to alum was administered mixed with saline (control) or with CPG 7909 at one of three doses (0.125, 0.5 or 1.0 mg). HBsAg-specific antibody responses (anti-HBs) appeared significantly sooner and were significantly higher at all timepoints up to and including 24 weeks in CPG 7909 recipients compared to control subjects ($p \leq 0.001$). Strikingly, most CpG 7909-vaccinated subjects developed protective levels of anti-HBs IgG within just two weeks of the priming vaccine dose. A trend towards higher rates of positive cytotoxic T cell lymphocyte responses was noted in the two higher dose groups of CPG 7909 compared to controls. The most frequently reported adverse events were injection site reactions, flu-like symptoms and headache. While these were more frequent in CPG 7909 groups than in the control group ($p < 0.0001$), most were reported to be of mild to moderate intensity regardless of group. In summary, CPG 7909 as an adjuvant to Engerix-B was well-tolerated and enhanced vaccine immunogenicity. CPG 7909 may allow the development of a two-dose prophylactic HBV vaccine.

KEY WORDS: Adjuvant; CpG oligodeoxynucleotide; HBV; vaccine; TLR9.

INTRODUCTION

Despite the availability of effective vaccine prophylaxis against the hepatitis B virus (HBV), acute clinical infections and their long-term sequelae remain important worldwide health concerns (1, 2). While current three-dose HBV vaccine schedules administered over 6 months are generally effective in healthy individuals (3), rapid induction of protective immunity, ideally after fewer doses, would be advantageous. Waiting 6 months until development of effective protection may expose health professionals to the continuing risk of infection. Furthermore, in endemic areas where infants are often infected through vertical or horizontal transmission, a rapidly effective vaccine is desirable considering that almost all infected infants become chronically infected (1, 2). As well, fewer vaccine doses would be more cost effective and would likely improve compliance. Adjuvants are frequently added to antigens in vaccine formulations to improve immune responses. Some, such as alum provide a depot effect, which improves antigen uptake and presentation by antigen presenting cells. Alum is safe and well tolerated but is relatively weak as an adjuvant and is strongly Th2 biased (4, 5). While Th2 adjuvants can induce high antibody titers, they are very poor for inducing cell-mediated immune responses such as cytotoxic T lymphocytes (CTL), which are essential for clearance of virus-infected cells. Oligodeoxynucleotides (ODN) that contain immunostimulatory CpG motifs (CpG ODN) are potent stimulants of B-cells and plasmacytoid dendritic cells

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(pDC) through binding to Toll-like receptor 9 (TLR9) (6). Through cytokine secretion from these cells, CpG ODN also indirectly activates a number of other types of immune cells. When combined with antigens, CpG ODN potently augment antigen-specific responses that are strongly Th1-biased (7–9). The CpG effects that promote antigen-specific responses include the direct activation of B cells to secrete more antibodies, improved antigen presentation by DC and secretion of Th1 cytokines that provide CD-40 ligand independent T-help (6, 10). Three classes of CpG ODN (A-Class, B-Class and C-Class) have been identified (11). All are effective as vaccine adjuvants, but the B-Class are particularly effective in activating B cells. The ability of B-Class CpG ODN to enhance vaccine immunogenicity has been demonstrated with a wide variety of antigens in animal models (10), including with HBsAg in mice by systemic (12–15) and mucosal (16) routes, and in non-human primates (17, 18). A human study recently reported enhanced immunogenicity of a commercial HBV vaccine with 1018 ISS (immune stimulatory sequence), a B-class CpG ODN (19). Herein we report results from a phase I trial where we evaluated the safety, tolerability and potent adjuvant effects of CPG 7909, a B-Class CpG ODN, when added to a commercial HBV vaccine.

METHODS

Study Design and Subject Selection

This double-blind controlled phase I study was conducted at the Toronto Western Hospital, Toronto, Canada and The Ottawa Hospital Clinical Investigation Unit, Ottawa, Canada. Ethical Review Board approval was obtained at each site, and each patient provided voluntary informed consent. Eligible subjects were healthy adult HBV vaccine naïve volunteers, aged 18–35, with no serologic evidence of previously resolved or current HBV infection. Other exclusion criteria included liver enzyme levels outside the normal range, chronic HIV or HCV infection, or receipt of blood products, vaccines, or immune globulin within two months of enrollment. Subjects receiving immune suppressive medication, and those diagnosed with an immune or autoimmune dysfunction were not considered for this study. Pregnant subjects were excluded, as were female subjects of child-bearing potential who were not willing to practice adequate birth control.

Vaccines

Subjects received an adult dose (1 mL) of Engerix-B® (GlaxoSmithKline, Rixensart, BE) that contains 20 μg of alum-adsorbed yeast-derived recombinant HBsAg. Experimental vaccines also contained 0.125, 0.5 or 1.0 mg CPG 7909 (sequence 5'-TCG TCG TTT TGT CGT TTT GTC GTT-3'), which was made with a wholly phosphorothioate backbone to render it nuclease resistant (Coley Pharmaceutical Group, Wellesley, MA). The CPG 7909 or vehicle control (saline) was added in a volume of 0.3 mL to ensure subjects and study personnel remained blinded to vaccine group. All vaccines were prepared by an unblinded pharmacist at the trial site and were administered within 48 h of mixing. Each subject received three intramuscular (IM) doses into the right deltoid muscle at 0, 4 and 24 weeks.

Experimental Groups

Subjects were enrolled sequentially into three cohorts according to dose level of CPG 7909. Within cohort, subjects were randomized to receive an experimental vaccine or control in a 3:1 ratio. Originally, it was planned to formulate the experimental vaccines with 0.5, 1.0 or 2.0 mg CPG 7909 and to have 16 subjects per cohort (12 experimental and 4 control). Due to a blood sample handling laboratory error in the first cohort leading to loss of some immunogenicity data, eight additional subjects (six with 0.5 mg CPG 7909 and two control) were added. After an interim analysis indicated that the 0.5 mg and the 1 mg doses of CPG 7909 were highly effective at improving anti-HBs responses, the intended 2 mg CPG 7909 dosing cohort was replaced with a 0.125 mg dose in an effort to find the lowest effective dose. A total of 56 subjects were immunized, 14 receiving control vaccines and 42 receiving experimental vaccines with CPG 7909 [0.5 mg ($n = 18$), 1.0 mg ($n = 12$), 0.125 mg ($n = 12$)].

Safety Evaluation

Adverse effects were identified by clinical safety evaluation at baseline and at the following times post administration: first dose—24 h, 2 weeks, and 4 weeks (just prior to second dose); second dose—24 h, 6, 8, 12, and 24 weeks (just prior to third vaccine dose); third dose—24 h, 26 and 48 weeks. Subjects also documented symptoms in a diary, which was evaluated at each visit. Laboratory tests including a complete blood count, serum chemistry, liver and renal function, and coagulation measures were performed at baseline, and weeks 2, 4, 6, 24 and 26. Rheumatological measures including CH50 (total complement function), anti-dsDNA antibody, rheumatoid factor, and anti-nuclear antibody titre (ANA) were measured at baseline and weeks 4, 8, and 26.

Immunogenicity—Humoral Response

To assess anti-HBs responses, blood samples were obtained at baseline and 2, 4, 6, 8, 12, 24, 26 and 48 weeks after the initial vaccine dosing. Samples were tested in triplicate at a commercial laboratory by the Abbott AUSAB-EIA® (Abbott, Abbott Park, IL, USA); anti-HBs titers were expressed in mIU/mL based on comparison with standards defined by the World Health Organization (WHO). Anti-HBs titers for total IgG, as well as the IgG1, IgG2, IgG3 and IgG4 isotypes in samples recovered at baseline, 6 or 8 and 26 weeks post prime were determined by endpoint dilution ELISA assay.

Immunogenicity—Cellular Responses

To assess HBsAg-specific cell-mediated immune responses, whole blood was collected from subjects at baseline and at weeks 4, 6, 8 and 26 weeks. Peripheral blood mononuclear cells (PBMC) were recovered from the whole blood on a Ficoll gradient and cryopreserved until required for HBsAg-specific CTL activity evaluation. CTL activity was performed after 5 days of *ex vivo* restimulation by ^{51}Cr release assay using HBsAg-derived peptide pulsed immortalized autologous B cells as stimulators and targets (Amersham Biosciences, Piscataway, NJ) (complete procedure available upon request). CTL assays were validated by the ratio of spontaneous release to maximum release. A CTL assay in which the ratio of spontaneous release to maximum release was $\geq 30\%$ was disqualified. At each E:T ratio the % specific lysis was calculated as described previously (12). Each of the following criteria had to be met to define a positive CTL: (i) % specific lysis of >10 (ii) greater than two-fold increase in specific lysis between the lowest effector to target cell (E:T) ratio and the highest E:T ratio, with evidence of incremental progression, and (iii) a positive CTL at 26 weeks and a negative CTL at some time point prior. Subjects were deemed incomplete if (i) they could not be classified as positive or negative due to an absence of data points or (ii) they were negative for one pool and did not have a legitimate data point for the other pool at 26 weeks.

Data Analysis

The analyses for safety and tolerability parameters were performed on all randomized subjects who received at least one dose of study medication and who had post-dose safety information. Control subjects receiving Engerix-B with saline in each of the three cohorts were combined. The proportion of adverse events between Engerix-B plus CPG 7909 recipients at each dose level and Engerix-B alone recipients was compared by Fisher's exact test. Immunogenicity results were analyzed using the ITT/attrition adjusted population. Thus, if any subject failed to receive all three doses of vaccine, immunogenicity parameters were analyzed for those time points up to and including the date of the first missed dose, but not for time points subsequent to that time. Anti-HBs titres measured in mIU/mL were expressed as geometric mean titres (GMT) for each group. All control subjects receiving Engerix-B with saline were combined. The differences between GMTs achieved at a given time point for each of the Engerix-B plus CPG 7909 groups and the Engerix-B control group were compared by Student's two-sided *t*-test. Differences between dose levels of CPG 7909 were not compared since they had been enrolled and randomized to different doses sequentially. The proportions of subjects achieving seroconversion (anti-HBs ≥ 1 mIU/mL), seroprotective (anti-HBs ≥ 10 mIU/mL) and high titers (anti-HBs ≥ 100 mIU/mL) in the combined CPG 7909 groups versus the control group were compared by Fisher's exact test. Antibody titers expressed as end-point dilution titers for total IgG, IgG1, IgG2, IgG3 and IgG4 were also expressed as GMT for groups and compared in the same way as anti-HBs in mIU/mL. The proportion of subjects with positive CTL per group were determined according to the methods previously described and are presented accordingly.

RESULTS

Population Characteristics

This study was conducted between April 1999 and June 2001. A total of 90 subjects were screened, of which 56 were enrolled and received at least one dose of vaccine. Of the 36 adult volunteers screened in Ottawa, two were anti-HBs positive, three withdrew consent prior to vaccination, and 31 eligible subjects with negative anti-HBs titres received vaccination. Of the 54 subjects screened in Toronto, 25 received at least one injection. Baseline characteristics and demographics were evenly distributed among the three dose cohorts, with the exception of gender (Table I). All but two subjects completed the study. One subject in the control group discontinued vaccination after becoming pregnant. A CPG 7909 recipient (0.5 mg) received only the first two doses due to highly elevated anti-HBs titres.

Safety and Tolerance

The proportion of subjects reporting at least one adverse event was similar for all groups ($>90\%$) (Table II) although the number of events reported was statistically

Table I. Patient Characteristics (Intent-To-Treat Population)

Parameter	Engerix-B	Engerix-B + 0.125 mg CPG	Engerix-B + 0.5 mg CPG	Engerix-B + 1.0 mg CPG	Significance*
Number enrolled	14	12	18	12	NS
Number completed	13	12	17	12	NS
Age (years) Mean ± SD	25.5 ± 5.4	25.9 ± 3.2	27.2 ± 5.8	24.5 ± 5.4	NS
Gender					
Male	8	4	5	11	NS
Female	6	8	13	1	NS
Race					
Asian	2	0	1	0	NS
Black	1	0	3	0	NS
Caucasian	11	12	14	12	NS

Note: NS: not significant, SD: standard deviation. * $p > 0.99$ in all cases.

significantly greater in subjects receiving either 0.5 or 1.0 mg CPG 7909 ($p < 0.0001$). Common non-serious adverse events included local injection site pain and erythema, as well as systemic symptoms such as pyrexia, flu-like symptoms and headache. These events occurred more frequently in those receiving CPG 7909 ($p < 0.0001$); all were characterized as mild to moderate intensity regardless of group. Following vaccine injection group mean oral temperature, blood pressure, and pulse rate remained stable from baseline to hour 1, as well as on day 2 and day 14. Mild pyrexia (fever defined as temperature ≥38.0°C) occurred more frequently in subjects receiving CPG 7909 0.5 mg (39%) or 1.0 mg (33%) than with 0.125 mg (8%). No subjects receiving Engerix-B alone reported fever ($p = 0.02$; compared to all/pooled CPG treated groups). One subject developed a hypersensitivity-type reaction with symptoms including warmth, weakness, nausea, and dizziness immediately following the third dose of Engerix-B plus 1.0 mg CPG 7909; the symptoms were transient and no medical or pharmacologic intervention was required. One subject receiving CPG 7909 1.0 mg was hospitalized for depression and suicidal ideation. This event was deemed unlikely to be related to exposure to the investigational drug due to a pre-existing condition.

Laboratory

There were no consistent or dose-related changes in biochemical or rheumatologic measures (data not shown). One CPG 7909 1.0 mg recipient demonstrated moderate elevations in anti-dsDNA values first detected 2 weeks after the 2nd and 3rd doses; these returned to normal prior to next dose and end of study respectively. This subject's ANA was negative throughout and the subject did not report symptoms associated with autoimmune disease.

Vaccine Immunogenicity

In each of three groups receiving CPG 7909, anti-HBs GMTs were statistically significantly higher than the GMT for control vaccine recipients for all time points up to and including week 24 (Fig. 1) ($p \leq 0.001$). After the third

Table II. Adverse Events Occurring in at Least 10% of Subjects

	Engerix-B	Engerix-B + 0.125 mg CPG	Engerix-B +0.5 mg CPG	Engerix-B + 1.0 mg CPG	Overall P-value[1]
n	14	12	18	12	—
At least one injection site reaction	6	10	17	12	<0.001
Injection site pain	6	9	17	12	<0.001
Injection site erythema	2	2	6	1	0.395
Malaise	1	0	7	1	0.020
Myalgia	0	3	5	2	0.160
Arthralgias	1	1	3	1	<0.001
Fatigue	3	4	3	5	0.448
Headache	3	6	9	6	0.313
Fever	0	1	7	4	0.017
Rigors	1	3	9	5	0.088
Nasopharyngitis	6	3	8	4	>0.3
Dizziness	1	0	2	3	>0.3
Feeling cold	1	1	2	2	>0.3
Diarrhea	1	1	4	0	>0.3

[1]Fisher's Exact Test.

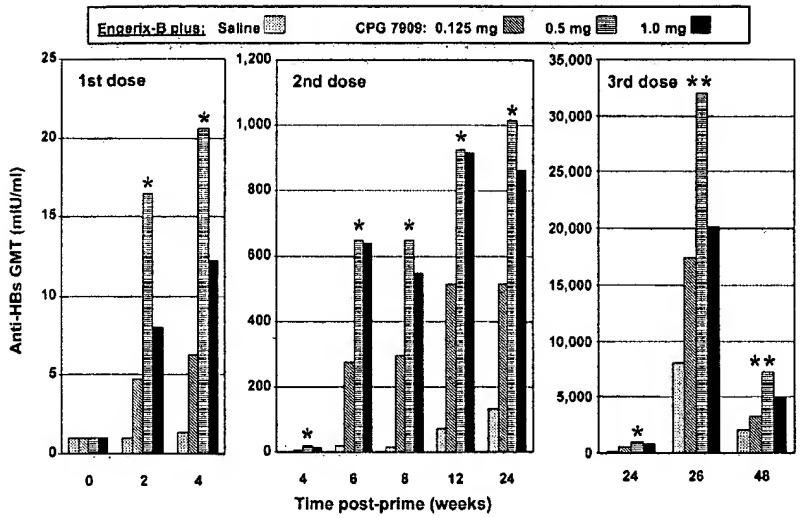

Fig. 1. Anti-HBs geometric mean titers (GMT) for control (Engerix-B + saline) and experimental (Engerix-B + 0.125mg, 0.5 mg or 1.0 mg CPG 7909) subjects (ITT with attrition) at each measured time-point during and after a 0, 4 and 24 week immunization schedule (arrow-heads). Each of the Engerix-B plus CPG 7909 groups was compared to Engerix-B + saline recipients by Student's two-tailed *t*-test. $^{*}p < 0.001$ for each CPG 7909 dose compared to control; $^{**}p < 0.05$ for 0.5 mg CPG compared to control.

dose at week 24, the GMT was significantly higher only for the CPG 7909 0.5 mg group compared to controls (Fig. 1). Addition of CPG 7909 to Engerix-B also resulted in significantly earlier appearance of anti-HBs. At two weeks post-prime, no subjects in the control group had detectable anti-HBs (0/14). In contrast, most subjects receiving Engerix-B plus CPG 7909 0.125 mg (6/12), 0.5 mg (10/12), or 1.0 mg (11/12) had seroconverted two weeks after prime ($p < 0.0001$ versus control). Furthermore, even at this very early time point, a high proportion CPG 7909 0.5 mg (7/12) and 1.0 mg (6/12) recipients and some 0.125 mg (4/12) recipients had attained seroprotective titers ($p = 0.015$ versus control) (Fig. 2). Post-boost (week 6) seroprotection was achieved in 100% of CPG 7909 recipients (0.5 mg 13/13; 1.0 mg 12/12; 0.125 mg 12/12) compared to 55% (6/11) of control

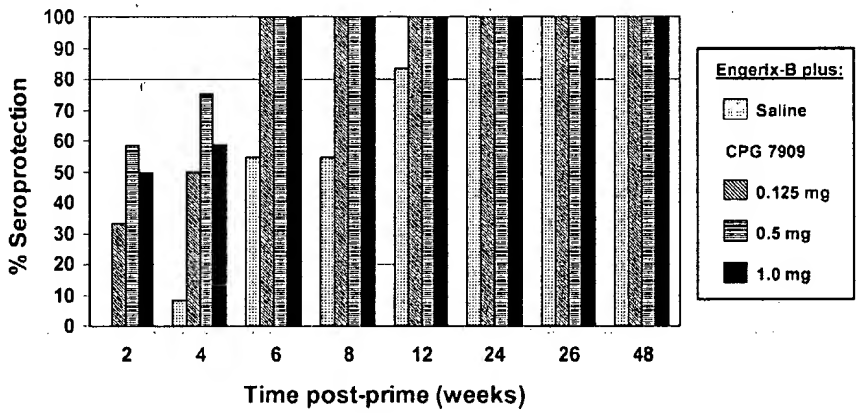

Fig. 2. The proportion of subjects attaining seroprotective anti-HBs titers (≥10 mIU/mL) for control (Engerix-B + saline) and experimental (Engerix-B + 0.125 mg, 0.5 mg or 1.0 mg CPG 7909) groups (ITT with attrition) at each measured time-point during and after a 0, 4 and 24 week immunization schedule. Combined Engerix-B plus CPG 7909 recipients (all doses) to Engerix-B plus saline controls were compared by Fisher Exact Test and results were significantly different ($p \leq 0.05$) at 2 to 12 weeks inclusive.

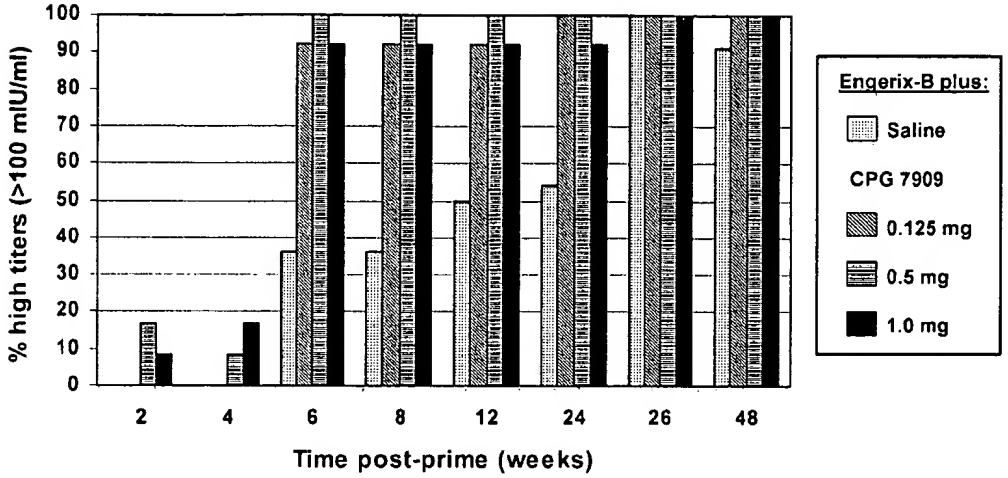

Fig. 3. The proportion of subjects obtaining high anti-HBs titers (≥100 mIU/mL) for control (Engerix-B + saline) and experimental (Engerix-B + 0.125 mg, 0.5 mg or 1.0 mg CPG 7909) groups (ITT with attrition) at each measured time-point during and after a 0, 4 and 24 week immunization schedule. Combined Engerix-B plus CPG 7909 recipients (all doses) to Engerix-B plus saline controls were compared by Fisher Exact Test and results were significantly different ($p \leq 0.05$) at 2 to 24 weeks inclusive.

subjects ($p = 0.0003$). Twelve months post prime, all subjects who had received the full course of vaccination maintained seroprotective anti-HBs titers. Statistically significantly more Engerix B-CPG 7909 subjects than controls had high-titre anti-HBs response (≥100 mIU/mL) as early as week 6 ($p = 0.0001$) and up to week 24 ($p = 0.0006$) (Fig. 3). Overall, the magnitude of antibody response appeared to be greater for the 0.5 and 1.0 mg CPG 7909 dose groups than the 0.125 mg group although this was not tested statistically since subjects were not randomized to dose of CPG (Fig. 1).

All groups had a similar profile for IgG antibody isotypes (Fig. 4). IgG1 was the dominant isotype detected, with moderately high levels of IgG3, and very low levels of IgG2 and IgG4. Titers of IgG1 isotype were statistically higher for each Engerix-B + CPG 7909 vaccine group than for the Engerix-B control group at 6 and 8 weeks ($p < 0.005$) and for the 0.5 and 1.0 mg CPG 7909 groups at 26 weeks ($p < 0.02$). Titers of IgG3 anti-HBs were statistically higher for each CPG 7909 group than for Engerix-B alone at 6 and 8 weeks and for the 0.5 and 1.0 mg CPG 7909 groups at 26 weeks ($p < 0.01$). Due to technical difficulties, HBsAg-specific CTL response data was not available for all subjects or at all time points. With the data that was available, no significant differences were detected for the proportion of subjects having positive CTL in the control versus CPG groups individually or combined ($p > 0.05$). Descriptively, there was a trend towards the higher doses of CPG 7909 recipients having positive CTL. One of seven (14%) Engerix-B control subjects had a positive CTL compared to for the CPG groups having 1/8 (12.5%) with 0.125 mg, 2 of 9 (22%) with 0.5 mg and 4/9 (44%) with 1 mg CPG 7909.

DISCUSSION

While current HBV vaccine strategies are generally satisfactory, they are not ideal for at-risk populations who require rapid protective immunity, such as health professionals, laboratory workers, and newborns to carrier mothers. As well, in developing areas of the world where HBV is often endemic, a two-dose regimen that could induce seroprotective responses within weeks rather than months would be advantageous both for economic and logistical reasons. Based on compelling animal data (18) and the need for improved HBV vaccine efficacy, we evaluated Engerix-B vaccine with and without CPG 7909 for safety and immunogenicity in this first human clinical study of CPG 7909. This CPG 7909 vaccine adjuvant was generally well tolerated, but the majority of subjects experienced at least one mild to moderate adverse event. Transient low-grade fever was noted in some subjects receiving CPG 7909 plus Engerix-B but not in subjects receiving vaccine alone. This effect is likely attributable to the immune stimulatory properties of CPG 7909. Flu-like symptoms were reported more frequently in subjects receiving 0.5 mg and 1.0 mg doses of CPG 7909 than in recipients of lower dose CPG 7909 or vaccine alone. Local injection site pain and headache were more frequently reported in recipients of

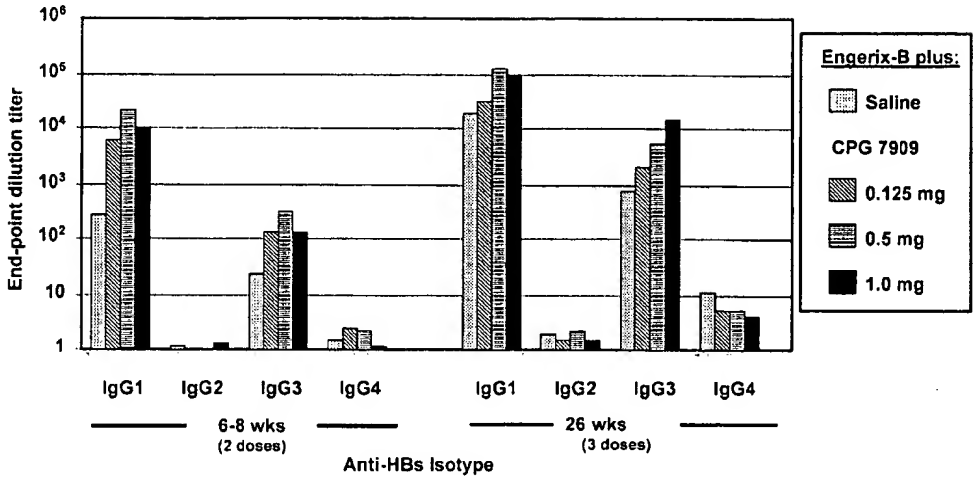

Fig. 4. Geometric means of end-point dilution titers (GMT) for different IgG isotypes of anti-HBs for control (Engerix-B + saline) and experimental (Engerix-B + 0.125 mg, 0.5 mg or 1.0 mg CPG 7909) groups (ITT with attrition) at each measured time-point during and after a 0, 4 and 24 week immunization schedule. Each Engerix-B plus CPG 7909 group was compared to the Engerix-B plus saline control group by Student's two-tailed *t*-test and for each of the four IgG isotypes the titers attained with all three doses of CPG 7909 was significantly greater than those induced with the control vaccine ($p < 0.02$).

Engerix-B-CPG 7909 vaccines but were short lived and did not limit daily activities. Local injection site symptoms are thought to be due to activation of local pro-inflammatory innate immune responses by CPG 7909. Local injection site pain and swelling have been reported with other humoral response enhancing adjuvants including monophosphoryl lipid A (MPL, Corixa) to Engerix-B (20, 21). Theoretical concerns have been raised about the potential for CpG ODN to evoke or unmask underlying autoimmune disease (10). Although a single subject receiving 1.0 mg CPG 7909 had transient low-titer elevations in anti-dsDNA titres, no symptoms or other evidence of autoimmune disease was noted in this subject or any others in this study. Hemodynamic collapse owing to activation of the complement cascade had been reported with very high doses (>10 mg/kg) of antisense and CpG ODN's during preclinical toxicity testing in monkeys (22). Such changes, which are known to be sequence independent and due to the synthetic phosphorothioate backbone of the ODN, were not expected nor observed with the CPG 7909 doses (0.002–0.016 mg/kg) evaluated in this study. Despite the relatively small group sizes in this phase I study, highly significant differences between Engerix-B plus CPG 7909 (all three doses tested) and Engerix-B alone were detected for most immunogenicity measures. There was a clear trend for the 0.5 and 1.0 mg doses to be superior to the 0.125 mg dose. These effective adjuvant doses in humans are much lower (0.007 mg/kg) for than doses given to mice [10 μg (0.5 mg/kg) (Weeratna and Davis, unpublished findings)]. Human results were much closer to those in young (10 kg) orangutans, a member of the ape family closely related to chimpanzees and humans, where testing of a more extensive dose response with HBsAg and alum showed 50 μg (0.005 mg/kg) to be a minimal optimal dose (17). Improvement in immunogenicity by use of CPG 7909 was most obvious for the humoral response, reflected both in the kinetics and strength of the response. Of particular note were very rapid seroconversion and seroprotection rates. The magnitude of the CpG effect on vaccine immunogenicity is readily appreciated when compared to two similarly designed controlled studies evaluating Engerix-B plus MPL in healthy volunteers aged 19–25 ($n = 12$ and 15 respectively, three dose regimen) (21) or aged 18–40 ($n = 50$ per group, 2 dose regimen) (20). MPL is a ligand for the TLR4 receptor, which is expressed on human myeloid dendritic cells and monocytes, in contrast to the pattern of TLR9 expression on plasmacytoid dendritic cells and B cells. MPL did result in improved immunogenicity with trends towards improved kinetics and magnitude. Even with MPL, however, seroconversion was slower and titers were lower compared to anti-HBs responses with CPG 7909. For example, MPL gave only slightly higher levels of seroprotection than the control vaccine at 4 weeks (38% versus 17%), which were markedly inferior to the results with CPG 7909 compared to its control at the same time-point (75% versus 8%). Furthermore, anti-HBs titers were not enhanced by MPL after the first or second vaccine dose. Titers were

significantly higher only after the third dose (GMT 3X control). In contrast, anti-HBs response was significantly higher (4-50X) with CPG 7909 at all time points up to 24 weeks. The GMT attained after three doses of Engerix-B with MPL was under 3000 mIU/mL whereas with CPG 7909 0.5 mg, >30,000 mIU/mL GMT was achieved. Other experimental adjuvants (GM-CSF or FLT-3 ligand) have failed to produce any significant effect on the kinetics or magnitude of the anti-HBs response compared to their respective control group (23–25). These results suggest that activation of plasmacytoid dendritic cells and B cells through TLR9 may provide a superior adjuvant effect for vaccines, compared with stimulating myeloid dendritic cells and B cells through TLR4. There has been one previously published report by Halperin *et al.* of a clinical study using a different CpG ODN sequence (1018 ISS) (19). It is difficult to compare results since in that trial the vaccine did not contain alum (control subjects received HBsAg only), and the vaccine was given on an alternate dosing schedule (0 and 8 weeks versus 0, 4 and 24 weeks in the present study). Nevertheless, if one compares 4 weeks post-prime responses, it appears that higher doses of 1018 ISS were required to detect significant adjuvant effects compared to CPG 7909. A seroprotective titer was found in 0, 0, 0, 25, 75 and 87.5% of subjects for doses of 0, 0.3, 0.65, 1 and 3 mg 1018 ISS respectively in Halperin *et al.* (19) compared to 8, 50 and 75% for 0, 0.125 and 0.5 mg CPG 7909 respectively in the present study. Anti-HBs GMT's at 4 weeks were 1, 1, 6, 25 and 207 for 0, 0.3, 0.65, 1 and 3 mg 1018 ISS respectively compared to 1, 6 and 12 for 0, 0.125 and 0.5 mg CPG 7909, respectively. It is difficult to say if the apparent greater effects in the present study are due to the CpG sequence or to the lack of alum in the 1018 ISS formulation.

Several possible limitations in this study are acknowledged. This investigation was a phase Ib/IIa study designed to evaluate the safety of CPG 7909 in healthy adults. It is possible that infrequent adverse events, not identified in this number of subjects, may subsequently be detected with further investigation. Long-term follow-up is required to determine if the higher titers with Engerix-CPG 7909 vaccines are sustained. The broad application of these results may be limited by the fact that healthy, adult volunteers were enrolled. However, another study of Engerix-B plus CPG 7909 in hyporesponsive HIV seropositive subjects achieved rapid, high, and sustained anti-HBs titres (manuscript in preparation). While the results from the CTL assays are encouraging, further study is necessary to determine whether CPG 7909 truly enhances cell-mediated immunity. If CpG ODN were able to enhance Th1 immunity in humans as it does in animal models, then evaluation of CpG-containing HBV vaccines for the treatment of HBV chronic infection would be justified. In summary, the addition of CPG 7909 to a HBV vaccine formulation comprising alum-absorbed HBsAg appears to be safe and well-tolerated. Furthermore, CPG 7909 may be more immunogenic than several other experimental adjuvants that have also been tested with HBV vaccines. Seroprotective anti-HBs levels were induced quickly and in a majority of subjects within 2 weeks, a time-point when the current commercial vaccines offer virtually no protection. This could allow rapid and effective protection of at-risk individuals. Furthermore, all subjects receiving Engerix-CPG 7909 vaccines had seroprotective titers with only two doses, and by 6 weeks. The possibility to reduce the number of required doses from three to two could greatly enhance compliance with the WHO directive for universal worldwide HBV immunization by allowing more favorable economic and logistical conditions.

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REFERENCES

1. Lee WM: Hepatitis B virus infection. N Engl J Med 337:1733–1745, 1997
2. Maddrey WC: Hepatitis B: An important public health issue. J Med Virol 61:362–366, 2000
3. Hepatitis B Vaccination–United States, 1982–2002, MMWR Morb Mortal Wkly Rep 51:549–552, 563, 2002
4. Chisari FV: Cytotoxic T cells and viral hepatitis. J Clin Invest 99:1472–1477, 1997
5. Rehermann B: Intrahepatic T cells in hepatitis B: Viral control versus liver cell injury. J Exp Med 191:1263–1268, 2000
6. Krieg AM: CpG motifs in bacterial DNA and their immune effects. Annu Rev Immunol 20:709–760, 2002
7. Chu RS, Targoni OS, Krieg AM, Lehmann PV, Harding CV: CpG oligodeoxynucleotides act as adjuvants that switch on T helper 1 (Th1) immunity. J Exp Med 186:1623–1631, 1997
8. Lipford GB, Bauer M, Blank C, Reiter R, Wagner H, Heeg K: CpG-containing synthetic oligonucleotides promote B and cytotoxic T cell responses to protein antigen: A new class of vaccine adjuvants. Eur J Immunol 27:2340–2344, 1997
9. Roman M, Martin-Orozco E, Goodman JS, Nguyen MD, Sato Y, Ronaghy A, Kornbluth RS, Richman DD, Carson DA, Raz E: Immunostimulatory DNA sequences function as T helper-1-promoting adjuvants. Nat Med 3:849–854, 1997

10. Krieg AM, Davis HL: Enhancing vaccines with immune stimulatory CpG DNA. Curr Opin Mol Ther 3:15–24, 2001
11. Vollmer J, Weeratna R, Payette P, Jurk M, Schetter C, Laucht M, Wader T, Tluk S, Liu M, Davis HL, Krieg AM: Characterization of three CpG oligodeoxynucleotide classes with distinct immunostimulatory activities. Eur J Immunol 34:251–262, 2004
12. Davis HL, Weeratna R, Waldschmidt TJ, Tygrett L, Schorr J, Krieg AM, Weeranta R: CpG DNA is a potent enhancer of specific immunity in mice immunized with recombinant hepatitis B surface antigen. J Immunol 160:870–876, 1998
13. Weeratna R, Comanita L, Davis HL: CPG ODN allows lower dose of antigen against hepatitis B surface antigen in BALBc mice. Immunol Cell Biol 81:59–62, 2003
14. Weeratna RD, Brazolot Millan CL, McCluskie MJ, Siegrist CA, Davis HL: Priming of immune responses to hepatitis B surface antigen in young mice immunized in the presence of maternally derived antibodies. FEMS Immunol Med Microbiol 30:241–247, 2001
15. Weeratna RD, McCluskie MJ, Xu Y, Davis HL: CpG DNA induces stronger immune responses with less toxicity than other adjuvants. Vaccine 18:1755–1762, 2000
16. McCluskie MJ, Davis HL: CpG DNA is a potent enhancer of systemic and mucosal immune responses against hepatitis B surface antigen with intranasal administration to mice. J Immunol 161:4463–4466, 1998
17. Davis HL, Suparto II, Weeratna RR, Jumintarto Iskandriati DD, Chamzah SS, Ma'ruf AA, Nente CC, Pawitri DD, Krieg AM, Heriyanto Smits W, Sajuthi DD: CpG DNA overcomes hyporesponsiveness to hepatitis B vaccine in orangutans. Vaccine 18:1920–1924, 2000
18. Hartmann G, Weeratna RD, Ballas ZK, Payette P, Blackwell S, Suparto I, Rasmussen WL, Waldschmidt M, Sajuthi D, Purcell RH, Davis HL, Krieg AM: Delineation of a CpG phosphorothioate oligodeoxynucleotide for activating primate immune responses in vitro and in vivo. J Immunol 164:1617–1624, 2000
19. Halperin SA, Van Nest G, Smith B, Abtahi S, Whiley H, Eiden JJ: A phase I study of the safety and immunogenicity of recombinant hepatitis B surface antigen co-administered with an immunostimulatory phosphorothioate oligonucleotide adjuvant. Vaccine 21:2461–2467, 2003
20. Ambrosch F, Wiedermann G, Kundi M, Leroux-Roels G, Desombere I, Garcon N, Thiriart C, Slaoui M, Thoelen S: A hepatitis B vaccine formulated with a novel adjuvant system. Vaccine 18:2095–2101, 2000
21. Thoelen S, Van Damme P, Mathei C, Leroux-Roels G, Desombere I, Safary A, Vandepapeliere P, Slaoui M, Meheus A: Safety and immunogenicity of a hepatitis B vaccine formulated with a novel adjuvant system. Vaccine 16:708–714, 1998
22. Galbraith WM, Hobson WC, Giclas PC, Schechter PJ, Agrawal S: Complement activation and hemodynamic changes following intravenous administration of phosphorothioate oligonucleotides in the monkey. Antisense Res Dev 4:201–206, 1994
23. West DJ: Clinical experience with hepatitis B vaccines. Am J Infect Control 17:172–180, 1989
24. Hasan MS, Agosti JM, Reynolds KK, Tanzman E, Treanor JJ, Evans TG: Granulocyte macrophage colony-stimulating factor as an adjuvant for hepatitis B vaccination of healthy adults. J Infect Dis 180:2023–2026, 1999
25. Evans TG, Hasan M, Galibert L, Caron D: The use of Flt3 ligand as an adjuvant for hepatitis B vaccination of healthy adults. Vaccine 21:322–329, 2002

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Safety and immunogenicity of CPG 7909 injection as an adjuvant to Fluarix influenza vaccine

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Abstract

CPG 7909, a 24-mer B-Class CpG oligodeoxynucleotide (ODN), was tested for safety, tolerability and its ability to augment the immunogenicity of a commercial trivalent killed split influenza vaccine (Fluarix® containing A/Beijing/262/95, A/Sydney/5/97 and B/Harbin/7/94; SmithKline Beecham) in a phase Ib blinded, randomized, controlled clinical trial. Sixty healthy volunteers were recruited in two consecutive cohorts of 30 subjects, who were randomly assigned to receive Fluarix plus 1 mg CPG 7909 or Fluarix plus saline control (15 subjects each). Vaccines were administered by intramuscular injection on a single occasion with subjects in the first cohort receiving a 1/10th dose of Fluarix and those in the second cohort receiving the full-dose. All safety measures including physical evaluation, laboratory blood assays, and assays for DNA autoimmunity were within normal values except for transient and clinically inconsequential decreases in total white blood cell counts in groups receiving CPG 7909. All vaccines were found to be generally well tolerated with similar frequency and intensity for most adverse reactions for groups receiving CPG 7909 as controls. Exceptions were injection site pain and headache, which were reduced in frequency in subjects receiving the 1/10th Fluarix dose without CpG, compared to the frequency in all other groups. There was a lack of pre-existing immunity, defined as hemagglutinin inhibition (HI) activity ≤20, for all subjects to the influenza strains A/Beijing/262/95 and B/Harbin/7/94 and for some subjects to A/Sydney/5/97. Post-vaccination humoral immune responses, as determined 2 and 4 weeks later by assay of HI activity and ELISA to detect antibodies against hemagglutinin (anti-HA) were similar for both full and reduced Fluarix doses but the cellular immune responses (measured as PBMC antigen-specific IFN-γ secretion) were reduced in the 1/10th Fluarix dose group. Humoral responses were not significantly enhanced by the addition of CPG 7909, except in individuals with pre-existing immunity to A/Sydney/5/97 strain (baseline HI activity titre >20), where there was a trend to higher HI activity with CPG 7909 ($P = 0.06$). The addition of CPG 7909 to the 1/10th dose of Fluarix did however result in significantly higher levels of IFN-γ secretion from peripheral blood mononuclear cells recovered at 4 weeks and restimulated ex vivo with A/Beijing/262/95 ($P = 0.048$) and B/Harbin/7/94 ($P = 0.0057$), restoring these to the level seen with full-dose vaccine. These results suggest that addition of CPG 7909 to Fluarix may allow the use of reduced vaccine doses without reduced immunogenicity.

Keywords: CpG oligodeoxynucleotides; Influenza; Vaccine; Adjuvant

1. Introduction

Oligodeoxynucleotides (ODN) containing immunostimulatory CpG motifs, which are unmethylated cytosine and guanosine dinucleotide pairs within the context of certain flanking sequences (CpG ODN) [1], can act as potent vaccine adjuvants [2–4]. CpG ODN directly activate B lymphocytes and plasmacytoid dendritic cells (pDC), the only cells known to express toll-like receptor 9 (TLR9) in humans [5] and also indirectly activate macrophages and other monocytes to secrete predominantly Th1-like cytokines and chemokines [6], which in turn can augment adaptive immune responses, as has been shown with a wide range of antigens [1–3,7–10]. In particular, CpG ODN enhances the development of T-helper type 1 (Th1)-type immune responses,

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including cytotoxic T lymphocytes (CTL) [1–3,6,9]. CPG 7909 is a 24-mer ODN containing three CpG motifs that is being developed for use in humans, including as a vaccine adjuvant.

Although 70–90% of influenza vaccinated children and young adults develop post-vaccination hemagglutination antibody (HA) titres that are associated with increased protection against infection by the same or closely similar strains of influenza virus [11–15], the level of protection is reduced for more distantly related strains [16–19]. In the elderly [19–21] and other relatively immune compromised populations [22,23], which are at risk for severe influenza disease, seroprotection against infection is also diminished, and antibody titres wane rapidly following vaccination [24]. Most influenza vaccines used currently do not contain adjuvants and their efficacy might be improved through the addition of an effective adjuvant. Alum is a commonly used adjuvant, and in most countries is the only one found in vaccines licensed for human use. Despite the increased immunogenicity it confers on other vaccines, alum has been found to be of limited use in combination with influenza vaccine [25,26]. Furthermore, alum results in a predominantly Th2-type response, with little or no CTL.

CpG ODN as a vaccine adjuvant has been found to be considerably more potent than alum in mice [2,27] that allows 10–100-times less antigen to be used [28] When used in combination with alum, CpG ODN can greatly enhance antibody responses to hepatitis B surface antigen in non-human primates [29,30] and humans [31]. CpG ODN have also been tested with influenza antigens in mice and found to be beneficial for increasing immunogenicity of the vaccine and reducing morbidity and mortality with subsequent viral challenge [7]. Addition of CPG 7909 to the Fluarix split-killed influenza vaccine induced significantly higher anti-HA antibody titres in mice receiving a single intramuscular (IM) dose [7]. In a protection and challenge study, mice receiving a whole killed influenza vaccine with alum and CpG ODN 1826 and then challenged with a mouse-adapted A strain of influenza virus (PR8) experienced less weight loss and no mortality in comparison to those receiving the vaccine with alum but without CpG ODN (Britta Wahren, personal communication). Anti-HA IgG titres were much higher in CpG ODN recipients as well.

Based on this preclinical data, we predicted that CPG 7909 would improve the immunologic response to an influenza vaccine. With this rational in mind, we have conducted a randomized controlled phase I study in healthy volunteers to assess the safety and immunogenicity of CPG 7909 with a commercial influenza vaccine (Fluarix®, SmithKline Beecham). Furthermore, based on the antigen-sparing effects of CpG ODN in animal models, we tested its immunogenicity with both a full-dose as well as a reduced dose (1/10th of the full-dose) of Fluarix.

2. Methods

2.1. Study design

This phase Ib, randomized, controlled, double-blind vaccine study was conducted at The Ottawa Hospital (General Campus) Clinical Investigation Unit, Ottawa, Canada. The study was reviewed and approved by The Ottawa Hospital Research Ethics Board. Healthy, consenting volunteers aged 18–40 years with negative HI titres to A/Beijing/262/95 and B/Harbin/7/94 were enrolled in two cohorts between August and December 1999.

2.2. Vaccines and experimental groups

Participants received commercially available Fluarix influenza vaccine (SmithKline Beecham Biologicals, Rixensart, Belgium, DIN# 37796), a trivalent killed split non-adjuvanted influenza vaccine. The vaccine used in this study comprised A/Beijing/262/95, A/Sydney/5/97, and B/Harbin/7/94 strains and had been used during the 1998–1999 influenza season in Europe. The usual full adult dose (0.5 ml undiluted) contained 15 μg of each hemagglutinin (HA) antigen and thus the 1/10th Fluarix dose contained 1.5 μg of each HA antigen.

CPG 7909, a B-Class CpG ODN of sequence 5′-TCGTCGTTTTGTCGTTTTGTCGTT-3′ was synthesized with a wholly phosphorothioate backbone (Coley Pharmaceutical Group, Wellesley MA, Lot# 183I0698). This ODN contains three copies of the GTCGTT motif that has been reported as optimal for stimulation of the human TLR9 receptor [6].

The Fluarix vaccine (the reduced dose was first diluted with PBS to have same volume as the full-dose, namely 0.5 ml) and the CPG 7909 (1 mg dissolved in 0.2 ml PBS) were mixed by a pharmacist within 4 h of administration to the subject. Control vaccines had 0.2 ml of PBS added to ensure all vaccines were of equal volume (0.7 ml) and appearance. Vaccines were administered by a single intramuscular injection into the left deltoid muscle, according to the vaccine manufacturer's instructions.

In the first cohort, 30 subjects were randomized to receive either 1/10th dose Fluarix with saline (control) or 1/10th dose Fluarix with CPG 7909 (experimental). In the second cohort, another 30 subjects were randomized to receive the full-dose Fluarix with saline (control) or the full-dose fluarix with CPG 7909 (experimental).

The Department of Pharmacy, who were in no other way involved with the design or conduct of this study, were responsible for randomization of subjects and preparation of the injection solutions.

2.3. Exclusion criteria

Exclusion criteria included prior receipt of any 1998–1999 influenza vaccine, hemagglutinin inhibition (HI) activity for serotype A/Beijing/262/95 or B/Harbin/7/94

greater than 1:20, and hypersensitivity or allergy to influenza vaccine. Receipt of blood products, any vaccine, or immunoglobulin within 1 month was not allowed. Candidates with HIV, taking immune suppressant medication, or with a history of autoimmune disease were not considered for this study. Pregnant subjects or those unwilling to practice birth control were excluded as well.

2.4. Safety evaluation

Adverse effects were identified by clinical safety evaluation at baseline (day 0), day 2, and weeks 2, 4 and 8 post-vaccination. Subjects also recorded symptoms in a diary, which was evaluated at each visit. Laboratory tests, which included a complete blood count, serum chemistry, liver and renal function, and coagulation measures were performed at baseline, day 2 and week 2. Tests for autoimmune reactivity included CH50 (hemolysis), anti-dsDNA antibody, rheumatoid factor (RF), and anti-nuclear antibody titre (ANA) and were measured at baseline and 8 weeks.

2.5. Immunogenicity evaluation

HI titres were measured at baseline and days 14, 28 and 59 post-vaccination. Total and IgG isotype (IgG1, IgG2, IgG3, and IgG4) HA levels were determined at baseline and week 8.

For assay of cellular immunity, fresh whole blood was collected at baseline and weeks 2, 4 and 8 in green top heparin tubes (BD Vacutainer, Franklin Lakes, NJ, USA) and transferred within 24 h to Coley Pharmaceutical Group's Clinical Laboratory, Ottawa, Canada for same day isolation of PBMC by density gradient centrifugation. The PBMC were resuspended in freezing media containing 20% DMSO, chilled to −80 °C by 1 °/min and stored in liquid nitrogen until analysis. Samples were thawed quickly at 37 °C and the average PBMC viability after freeze/thawing was 82 ± 16% (mean ± S.D., $n = 110$). The cells were stimulated individually with each antigen, A/Sydney, A/Beijing and B/Harbin (generously provided by SmithKline Beecham Pharma GmbH, SSW Branch, Germany) at a concentration of 0.5 μg/ml for 5 days at 37 °C. Media was also included as a negative control and phytohemmagglutinin (PHA, 10 μg/ml, Sigma, St. Louis, MO, USA) and tetanus toxoid (1.0 μg/ml, List Biologicals, Campbell, USA) as positive controls. Cells were pulsed with ^{3}H-thymidine (10 μCi/ml, AMersham Health Sciences, Oakville, ON, Canada) for 16 h, harvested and measured for radioactivity. The stimulation index is the count/min (CPM) of cells incubated with antigen divided by the CPM of cells incubated with media alone. Production of IFN-α and IFN-γ was measured by antigen re-stimulation of PBMCs harvested at baseline and weeks 2 and 4.

2.6. Statistical analysis

Baseline characteristics of the groups were compared by Chi-square test, χ^2 for categorical variables and by Student's t-test for continuous variables. For safety and tolerance evaluation, data were assessed by χ^2 or Fisher's Exact Test for within Fluarix-dose cohort comparisons and by Mantel–Haenszel controlling for cohort. Hematological, biochemical, and immunological measures were compared by repeat measures ANOVA between CPG 7909 and placebo control recipients for each Fluarix-dose cohort. For each Fluarix-dose cohort the change from baseline in natural log HI titre was compared between CPG 7909 and placebo control recipients by Student's t-test and repeat measures ANOVA (for each of the three stains of antigen separately). HA antibody titre production were converted to fold increase from baseline and compared between Fluarix-dose cohorts by repeat measures ANOVA. T-cell proliferation stimulation index and cytokine (pg/ml) change from baseline were evaluated by Student's t-test (for each of the three stains of antigen separately). For all measures, differences were considered to be not significant with $P > 0.05$.

3. Results

3.1. Population characteristics

Ninety of 125 subjects initially screened were eligible for enrollment by virtue of having HI activity ≤20 for A/Beijing/262 and B/Harbin/7, and 60 of these subjects were actually vaccinated. The other 30 were not vaccinated because they declined vaccination ($n = 16$), withdrew consent ($n = 3$), could not be located ($n = 5$) or were ineligible for other reasons ($n = 6$). Active vaccination occurred from August to October 1999. All enrolled subjects completed the study (Table 1).

3.2. Safety and tolerance

Most study subjects reported at least one adverse event during the follow-up period, but only one event was classified as serious. In this case, a female subject who had received the 1/10th dose Fluarix (without CPG 7909) developed polyarthritis 1 month following the vaccine administration, and this was subsequently diagnosed and treated as seronegative rheumatoid arthritis.

There was no difference in frequency between subjects receiving control vaccines (either dose) or vaccines containing CPG 7909 for most common minor adverse events, including fever, fatigue, myalgia, arthralgia, as well as injection site bruising, erythema, edema and pruritus (Table 2). Only injection site pain and headache occurred more frequently in CPG 7909 recipients ($P = 0.004$ and 0.027, respectively) however this could be attributed solely to differences within the 1/10th dose cohort (Table 2). The duration of injection

Table 1
Subject characteristics

Parameter	1/10th dose Fluarix	1/10th dose Fluarix-CPG 7909	P	Full-dose Fluarix	Full-dose Fluarix-CPG 7909	P
N	15	15	–	14	16	–
Completed	15	15	–	14	16	–
Age mean (S.D.[a])[b]	27.3 (7.0)	29.9 (7.0)	NS	26.1 (4.1)	28.0 (5.2)	NS
Male sex[c]	9	9	NS	3	8	NS
A/Sydney HI > 20[c]	8	7	NS	8	10	NS

[a] Standard deviation.
[b] Student's *t*-test.
[c] Chi-square.

site pain was one to two days longer in CPG 7909 recipients. The intensity of injection site pain was graded as mild to moderate by all subjects with one exception; one female receiving the 1/10th dose Fluarix plus CPG 7909 reported severe injection site pain not associated with bruising or erythema that resolved completely by 4 days post-injection.

Local or generalized lymphadenopathy and hepatosplenomegaly were not identified in any vaccine recipients whether or not they received CPG 7909. Recorded oral temperature, blood pressure, and pulse rate were unchanged from baseline 1 h, 2 days, and 14 days following injection. A single subject reported influenza-like symptoms (full-dose Fluarix plus CPG 7909) and three individuals reported fever (1/10th dose Fluarix, $n = 1$; 1/10th Fluarix plus CPG 7909, $n = 2$) following injection. Symptoms and signs of upper respiratory infection and diagnosis of infections in follow-up did not differ between groups receiving CPG 7909 versus placebo control in each Fluarix-dose cohort.

3.3. Laboratory

In all four study arms, clinically insignificant and transient reductions in total white blood cell count, neutrophils, lymphocytes, eosinophils and platelets as well as an increase in monocytes were noted in blood drawn 2 days post-vaccination. These changes were not significantly different between subjects receiving control vaccines and those receiving vaccines with CPG 7909 except for decreases in total white blood cell count ($-1690\ \mu l^{-1}$ versus $-280\ \mu l^{-1}$ for CPG 7909 versus control in the 1/10th dose cohort, $P < 0.001$, and $-2350\ \mu l^{-1}$ versus $-1370\ \mu l^{-1}$ in the full-dose cohort, $P = 0.04$). These changes were clinically inconsequential and average values for all measures returned to baseline levels by day 14. No significant change from baseline in INR or PTT was observed in any group at any time. There were no apparent clinical consequences of any hematologic laboratory changes.

There were no significant changes in electrolytes, liver studies, renal function, serum glucose, lipids, and muscle enzymes (data not shown). Severe grade laboratory measures were not observed in any patient.

3.4. Immunopathology results

No significant changes in anti-ds DNA, total complement, rheumatoid factor and ANA were observed in any subject. Arthralgia, joint effusion, joint stiffness or arthritis were not reported in any CPG 7909 recipients.

3.5. Vaccine immunogenicity

The HI activity titers produced with both the 1/10th dose and full-dose Fluarix vaccines were significantly increased from baseline for all four vaccine groups at all post-vaccination time points measured for all three antigens and all subjects achieved HI activity titres ($\geq$1:40) to all three influenza antigens, a titer that is associated with increased protection against influenza infection (Table 3). Since some subjects had pre-existing HI activity against A/Sydney/5/97 antigen, post-vaccination titers were evaluated relative to

Table 2
Injection-related adverse events

	1/10th dose Fluarix	1/10th dose Fluarix-CPG 7909	P[a]	Full-dose Fluarix	Full-dose Fluarix-CPG 7909	P[a]	Overall P[b]
N	15	15		14	16		
At least one adverse event	11	14	0.142	12	16	0.118	0.040
Injection site pain	3	13	<0.001	11	13	0.855	0.004
Headache	1	6	0.029	3	6	0.200	0.027
Arthralgias	1	0	0.500	0	0	–	0.317
Myalgias	1	3	0.250	0	3	0.140	0.057
Fatigue	0	3	0.112	6	3	0.120	0.844
Fever	1	1	0.517	0	1	0.530	0.585

[a] Chi-square/Fisher's exact test.
[b] Cochran Mantel–Haeszel.

Table 3
Summary and comparison of change of natural log hemagglutination inhibition (HI) titres

Time point	1/10th dose Fluarix	1/10th dose Fluarix-CPG 7909	t-test[c]	Full-dose Fluarix	Full-dose Fluarix-CPG 7909	t-test[c]
(a) For A/Sydney/5/97 antigen from baseline in subjects with screening HI ≤ 20[a,b]						
N	7	8		6	6	
Day 14	2.27 (0.49)	1.86 (0.35)	0.50	3.88 (0.42)	2.79 (0.47)	0.12
Day 28	2.06 (0.52)	1.78 (0.34)	0.65	3.72 (0.43)	2.98 (0.53)	0.31
Day 56	1.92 (0.51)	1.47 (0.35)	0.47	3.58 (0.45)	2.93 (0.64)	0.42
(b) For A/Sydney/5/97 antigen from baseline in subjects with screening HI > 20[a]						
N	8	7		8	10	
Day 14	1.09 (0.33)	1.92 (0.28)	0.08[d]	1.97 (0.27)	2.58 (0.25)	0.12[d]
Day 28	1.21 (0.34)	2.08 (0.26)	0.07[d]	1.91 (0.32)	2.44 (0.23)	0.18[d]
Day 56	1.23 (0.39)	2.22 (0.27)	0.06[d]	1.77 (0.34)	2.45 (0.28)	0.14[d]
(c) For A/Beijing/262/95 antigen from baseline[a,b]						
N	15	15		14	16	
Day 14	2.44 (0.46)	2.85 (0.37)	0.49	3.14 (0.44)	3.22 (0.38)	0.88
Day 28	2.50 (0.49)	3.07 (0.37)	0.36	2.90 (0.42)	2.99 (0.32)	0.87
Day 56	2.48 (0.47)	3.16 (0.38)	0.28	2.71 (0.44)	2.92 (0.34)	0.70
(d) For B/Harbin/7/94 antigen from baseline[a,b]						
N	15	15		14	16	
Day 14	1.78 (0.23)	2.00 (0.29)	0.56	2.24 (0.40)	2.27 (0.28)	0.95
Day 28	1.95 (0.25)	1.99 (0.32)	0.91	2.03 (0.39)	2.05 (0.31)	0.96
Day 56	1.84 (0.24)	1.95 (0.34)	0.81	1.86 (0.39)	1.85 (0.30)	0.99

[a] Value expressed as change in natural log HI (standard error of the mean) from baseline.
[b] 1:20 HI titre = 3.00 ln.
[c] Interaction term not significant by repeated measures ANOVA analysis.
[d] $P = 0.06$ between Fluarix-CPG 7909and Fluarix-control groups.

baseline titres (Table 3a and 3b). For the other two antigens, A/Beijing/262/95 and B/Harbin/7/94, absolute titers were used since all subjects were seronegative prior to vaccination. Subjects receiving CPG 7909 did not attain higher HI titers than control subjects in the same vaccine dose cohort for any antigen (Table 3; $P > 0.05$). However, in subjects where there was pre-existing immunity against A/Sydney/5/97 there was a trend toward greater titre increase with CPG 7909 in the 1/10th Fluarix cohort ($P = 0.06$–0.08 at different times, Table 3b).

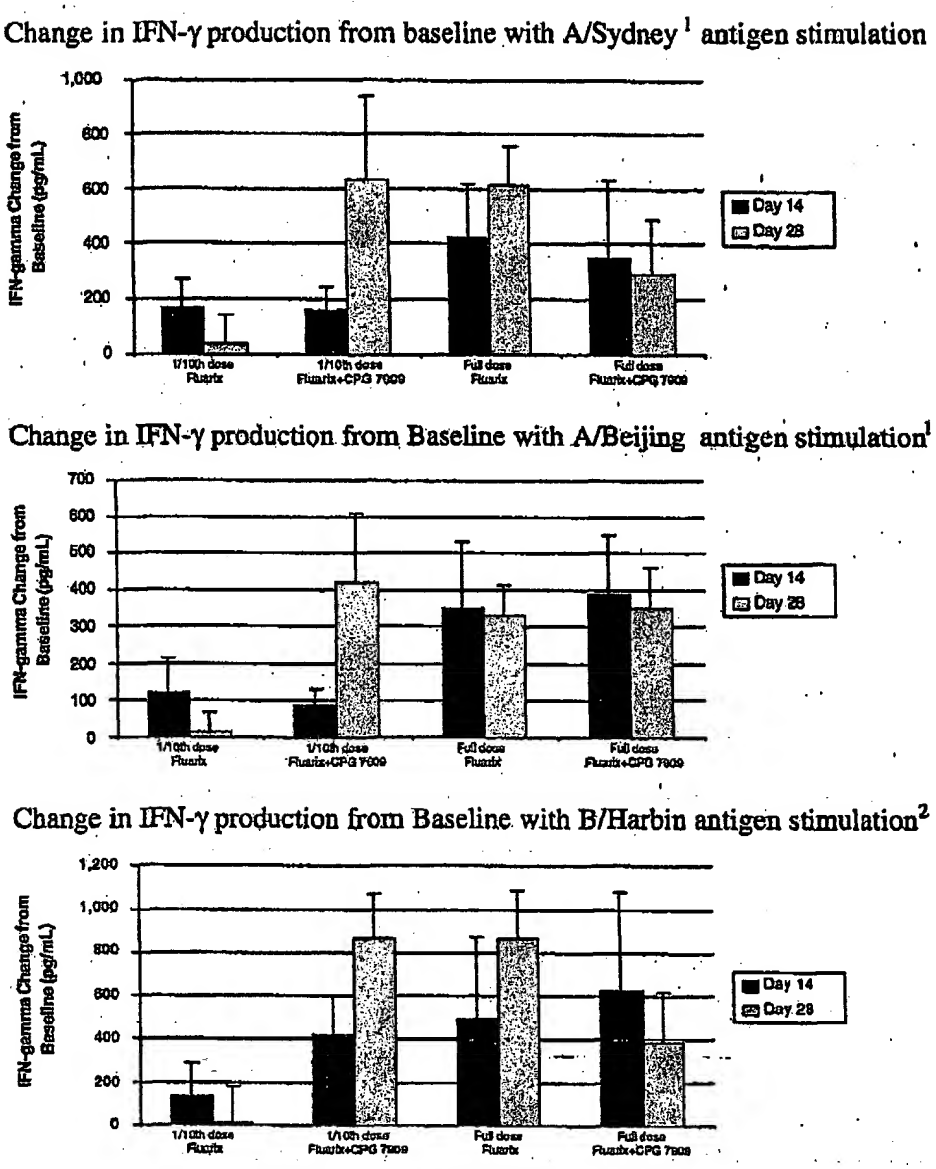
When anti-HA ELISA titers were determined, there were four and two-fold increases in total IgG and IgG1 against A/Sydney/5/97 at day 56 for full-dose and 1/10th dose Fluarix, respectively, regardless of CPG 7909 inclusion. IgG2 and IgG4 anti-HA titres to A/Sydney/5/97 were unchanged from baseline irrespective of Fluarix dose or inclusion of CPG 7909. A trend was noted toward greater fold increase in IgG3 anti-HA titre to A/Sydney/5/97 in recipients of full-dose Fluarix plus CPG 7909 versus full-dose Fluarix alone (10.4 versus 2.3-fold). This was most pronounced in those who had baseline A/Sydney/5/97 HI activity greater than 1:20. A trend toward greater fold increase in IgG3 anti-HA titres to A/Beijing/262/95 antigen was also noted in full-dose and 1/10th dose Fluarix plus CPG 7909 recipients when compared to their respective controls (44.1-fold versus 4.5-fold and 10.4-fold versus 1.9-fold, respectively). The mean fold increase in total IgG, IgG1, IgG2, and IgG4 anti-HA titres to A/Beijing/262/95 and B/Harbin/7/94 antigens at day 56 was not influenced by CPG 7909 inclusion and were similar between doses of Fluarix.

PBMC production of IFN-α and IFN-γ on ex vivo re-stimulation with any of the three influenza antigens was evaluated at baseline, day 14, and day 28. Subjects receiving CPG 7909 with the 1/10th vaccine dose had significantly higher IFN-γ production in response to A/Beijing/262/95 ($P = 0.048$) and B/Harbin/7/94 ($P = 0.0057$) antigens but not to the medium control (Fig. 1). A four to seven-fold increase in IFN-γ production was noted in full-dose Fluarix recipients, and this did not differ for antigen or CPG 7909 inclusion. No group had increased levels of IFN-α secretion with any antigen post-vaccination regardless of dose of vaccine or inclusion of CPG 7909 (data not shown).

PBMC proliferation increased by at least two-fold, irrespective of antigen, Fluarix dose, or CPG 7909 inclusion (data not shown). Although not statistically significant, the mean fold increase in PBMC proliferative responses to A/Beijing/262/95 in CPG 7909 recipients appeared to be greater at both at days 14 and 28 than controls in the 1/10th dose Fluarix cohort.

4. Discussion

In the present study, CPG 7909 was found to be safe and well tolerated when used as an adjuvant to the Fluarix influenza vaccine in healthy, adult recipients. Most adverse reactions were of similar frequency and magnitude between



[1]Significant difference between 1/10th dose Fluarix plus CPG 7909 and 1/10th dose Fluarix plus control on day 28 by Student's t test (p=0.048)
[2]Significant difference between 1/10th dose Fluarix plus CPG 7909 and 1/10th dose Fluarix plus control on day 28 by Student's t test (p=0.0057)

Fig. 1. T cell proliferation following stimulation with influenza antigens. (a) Change in IFN-γ production from baseline with A/Sydney[1] antigen stimulation. (b) Change in IFN-γ production from baseline with A/Beijing antigen stimulation.[1] (c) Change in IFN-γ production from baseline with B/Harbin antigen stimulation.[2]

CPG 7909 and control subjects receiving the same vaccine dose (full or 1/10th reduced dose). Only local injection site pain and headache occurred more frequently, but not with greater severity, in subjects receiving CPG 7909 and this was detected only with the 1/10th dose of vaccine. All adverse events were short lived and did not limit the daily activities of subjects. Theoretical concerns have been raised about the potential of CpG ODN to evoke or unmask underlying autoimmune disease [6]. No symptomatic or biochemical evidence of this was observed. Hemodynamic adverse effects,

as had been noted with administration of very high doses of antisense ODN to monkeys [32], were not seen in any of the 31 adult humans receiving CPG 7909 with Fluarix.

We predicted that CPG 7909 would increase the proportion of subjects achieving seroprotective HI titres as well as the actual titers. However, all subjects responded well to the vaccine alone, and no adjuvant effects by CPG 7909 on HI titers or anti-HA ELISA titers (various IgG isotypes) were noted with the exception of a trend towards higher titers against A/Sydney/5/97 in individuals with pre-existing immunity to A/Sydney/5/97. One interpretation of these results is that CPG 7909 may be better able to boost than prime responses to influenza antigens. Alternatively, since the vaccine alone turned out to be highly effective in this population, it is possible that a protective response to the influenza antigens was already being made, and that further boosting would not be beneficial. It is possible that CPG 7909 would have shown benefit if evaluated in vaccine hyporesponsive populations, such as the very young or elderly. The addition of CpG ODN to murine vaccines enables effective vaccination of neonatal and elderly mice [33–35]. Two doses of influenza vaccine with CPG 7909 may be effective in achieving high rates of protective immunity against influenza, especially in hypo-responsive populations. Evaluation of this approach is warranted. It may be that the formulation was not optimal in the present study where there was no other adjuvant to offer a depot effect [27,36,37]. Alternatively, it is possible that some characteristic of the immune response to the influenza vaccine in healthy subjects prevents it from being enhanced by the addition of an adjuvant. It is noteworthy that we have been unable to find any published reports of an adjuvant that enhances influenza responses in normal subjects.

Overall, the adjuvant effect of CPG 7909 was most apparent in the 1/10th vaccine dose cohort. One explanation for this may be that the full-dose of vaccine on its own is highly immunogenic with little room for improvement, but that the CPG 7909 compensates for the lower immunogenicity of the reduced amount of antigen. This would be consistent with our earlier results in mice, where the addition of CpG to a vaccine enabled effective vaccination with 1/100th of the normal antigen dose [28].

Based on its in vitro immune stimulation effects, we anticipated that CPG 7909, a B-Class CpG ODN, would enhance activation of B-cells. The most predominant immunoglobulin subclasses produced following vaccination in this study were IgG1 and IgG3. A trend toward greater increase in the IgG3 subclass to A/Sydney5/97 and A/Beijing/262/95 in Fluarix plus CPG 7909 recipients was noted at day 56. This may be clinically relevant as complement activation and antibody-dependent cell-mediated cytotoxicity responses, both important in viral neutralization, are most efficiently produced by IgG1 and IgG3 subclass antibodies [38,39]. We expected pDC stimulated by CPG 7909 to induce increased Th1 T cells, leading to enhanced antigen-specific IFN-γ secretion. Indeed, significantly higher levels of IFN-γ were detected upon restimulation of PBMCs with influenza antigens in subjects receiving CPG 7909 than in controls in the 1/10th dose Fluarix cohort. These findings may be clinically important as endogenous interferons have an important role in terms of decreased viral titres and symptom resolution [40–42].

Several limitations of this study are acknowledged. This phase Ib study was designed primarily to evaluate the safety of CPG 7909 as a vaccine adjuvant in humans. It is possible that infrequent adverse events, not identified in this small study, may be detected with future larger scale investigations. Of course, long term follow-up to detect protection from natural infection and degree of clinical illness with influenza are also required in the evaluation of this adjuvant for influenza vaccines such as Fluarix. The sequential enrollment into reduced and then full-dose vaccine cohorts limited our ability to perform direct statistical comparisons between these two doses of influenza vaccine. Enrolled subjects were purposely selected to have sub-seroprotective HI activity titres to A/Beijing and B/Harbin at baseline and therefore represent a subgroup of the general population. Nevertheless, 90 of 125 (72%) subjects screened met these criteria, indicating that this subgroup was at least representative of the majority of healthy adults living in the Ottawa area at that time. The relevance of this study to populations more likely to be hyporesponsive to an influenza vaccine or to be at greater risk of severe influenza may be limited by the fact that only healthy, adult volunteers were enrolled in this study. Further studies in these target populations are warranted.

In conclusion, this study demonstrates the safety of CPG 7909 as an adjuvant to a commercial influenza vaccine in healthy human volunteers. The effects of CPG 7909 on the immunogenicity of the vaccine were limited and might become more apparent by testing in an immune compromised population, by using a two-dose strategy or through improved formulation. Of note but unrelated to the testing of CPG 7909 was the finding that the 1/10th dose of Fluarix produced similarly high proportions of seroprotective antibody titers, as did full-dose vaccine. In contrast, antigen-specific IFN-γ production was reduced in the group receiving the 1/10th dose, but was restored to approximately the same level seen in the full-dose group by the addition of CPG 7909. This warrants further evaluation as it could be of relevance in situations where vaccine demand exceeds supply owing to fears of increased infection rates or virulence in a particular influenza season.

References

[1] Krieg AM, Yi AK, Matson S, Waldschmidt TJ, Bishop GA, Teasdale R, et al. CpG motifs in bacterial DNA trigger direct B-cell activation. Nature 1995;374:546–9.

[2] Davis HL, Weeratna R, Waldschmidt TJ, Tygrett L, Schorr J, Krieg AM, et al. CpG DNA is a potent enhancer of specific immunity in mice immunized with recombinant hepatitis B surface antigen. J Immunol 1998;160:870–6.

[3] Roman M, Martin-Orozco E, Goodman JS, Nguyen MD, Sato Y, Ronaghy A, et al. Immunostimulatory DNA sequences function as T helper-1-promoting adjuvants. Nat Med 1997;3:849–54.
[4] Lipford GB, Bauer M, Blank C, Reiter R, Wagner H, Heeg K. CpG-containing synthetic oligonucleotides promote B and cytotoxic T cell responses to protein antigen: a new class of vaccine adjuvants. Eur J Immunol 1997;27:2340–4.
[5] Ahmad-Nejad P, Hacker H, Rutz M, Bauer S, Vabulas RM, Wagner H. Bacterial CpG-DNA and lipopolysaccharides activate Toll-like receptors at distinct cellular compartments. Eur J Immunol 2002;32:1958–68.
[6] Krieg AM. CpG motifs: the active ingredient in bacterial extracts. Nat Med 2003;9:831–5.
[7] Moldoveanu Z, Love-Homan L, Huang WQ, Krieg AM. CpG DNA, a novel immune enhancer for systemic and mucosal immunization with influenza virus. Vaccine 1998;16:1216–24.
[8] Deml L, Schirmbeck R, Reimann J, Wolf H, Wagner R. Immunostimulatory CpG motifs trigger a T helper-1 immune response to human immunodeficiency virus type-1 (HIV-1) gp 160 envelope proteins. Clin Chem Lab Med 1999;37:199–204.
[9] Hartmann G, Weiner GJ, Krieg AM. CpG DNA: a potent signal for growth, activation, and maturation of human dendritic cells. Proc Natl Acad Sci USA 1999;96:9305–10.
[10] Weiner GJ, Liu HM, Wooldridge JE, Dahle CE, Krieg AM. Immunostimulatory oligodeoxynucleotides containing the CpG motif are effective as immune adjuvants in tumor antigen immunization. Proc Natl Acad Sci USA 1997;94:10833–7.
[11] Palache AM. Influenza vaccines. A reappraisal of their use. Drugs 1997;54:841–56.
[12] Meiklejohn G, Eickhoff TC, Graves P, I J. Antigenic drift and efficacy of influenza virus vaccines, 1976–1977. J Infect Dis 1978;138:618–24.
[13] Ruben FL. Prevention and control of influenza. Role of vaccine. Am J Med 1987;82:31–4.
[14] Hirota Y, Kaji M, Ide S, Kajiwara J, Kataoka K, Goto S, et al. Antibody efficacy as a keen index to evaluate influenza vaccine effectiveness. Vaccine 1997;15:962–7.
[15] Bridges CB, Fukuda K, Cox NJ, Singleton JA. Prevention and control of influenza. Recommendations of the Advisory Committee on Immunization Practices (ACIP). MMWR Recomm Rep 2001;50:1–44.
[16] Meiklejohn G. Viral respiratory disease at Lowry Air Force Base in Denver, 1952–1982. J Infect Dis 1983;148:775–84.
[17] Sugaya N, Nerome K, Ishida M, Matsumoto M, Mitamura K, Nirasawa M. Efficacy of inactivated vaccine in preventing antigenically drifted influenza type A and well-matched type B. JAMA 1994;272:1122–6.
[18] Barker WH, Mullooly JP. Influenza vaccination of elderly persons. Reduction in pneumonia and influenza hospitalizations and deaths. JAMA 1980;244:2547–9.
[19] Ruben FL, Johnston F, Streiff EJ. Influenza in a partially immunized aged population. Effectiveness of killed Hong Kong vaccine against infection with the England strain. JAMA 1974;230:863–6.
[20] Govaert TM, Thijs CT, Masurel N, Sprenger MJ, Dinant GJ, Knottnerus JA. The efficacy of influenza vaccination in elderly individuals. A randomized double-blind placebo-controlled trial. JAMA 1994;272:1661–5.
[21] Gross PA, Hermogenes AW, Sacks HS, Lau J, Levandowski RA. The efficacy of influenza vaccine in elderly persons. A meta-analysis and review of the literature. Ann Intern Med 1995;123:518–27.
[22] Blumberg EA, Albano C, Pruett T, Isaacs R, Fitzpatrick J, Bergin J, et al. The immunogenicity of influenza virus vaccine in solid organ transplant recipients. Clin Infect Dis 1996;22:295–302.
[23] Dorrell L, Hassan I, Marshall S, Chakraverty P, Ong E. Clinical and serological responses to an inactivated influenza vaccine in adults with HIV infection, diabetes, obstructive airways disease, elderly adults and healthy volunteers. Int J STD AIDS 1997;8:776–9.
[24] Buxton JA, Skowronski DM, Ng H, Marion SA, Li Y, King A, et al. Influenza revaccination of elderly travelers: antibody response to single influenza vaccination and revaccination at 12 weeks. J Infect Dis 2001;184:188–91.
[25] Skea DL, Barber BH. Adhesion-mediated enhancement of the adjuvant activity of alum. Vaccine 1993;11:1018–26.
[26] Powers DC, Smith GE, Anderson EL, Kennedy DJ, Hackett CS, Wilkinson BE, et al. Influenza A virus vaccines containing purified recombinant H3 hemagglutinin are well tolerated and induce protective immune responses in healthy adults. J Infect Dis 1995; 171:1595–9.
[27] Weeratna RD, McCluskie MJ, Xu Y, Davis HL. CpG DNA induces stronger immune responses with less toxicity than other adjuvants. Vaccine 2000;18:1755–62.
[28] Weeratna R, Comanita L, Davis HL. CPG ODN allows lower dose of antigen against hepatitis B surface antigen in BALB/c mice. Immunol Cell Biol 2003;81:59–62.
[29] Davis HL, Suparto II, Weeratna RR, Jumintarto, Iskandriati DD, Chamzah SS, et al. CpG DNA overcomes hyporesponsiveness to hepatitis B vaccine in orangutans. Vaccine 2000;18:1920–4.
[30] Hartmann G, Weeratna RD, Ballas ZK, Payette P, Blackwell S, Suparto I, et al. Delineation of a CpG phosphorothioate oligodeoxynucleotide for activating primate immune responses in vitro and in vivo. J Immunol 2000;164:1617–24.
[31] Halperin SA, Van Nest G, Smith B, Abtahi S, Whiley H, Eiden JJ. A phase I study of the safety and immunogenicity of recombinant hepatitis B surface antigen co-administered with an immunostimulatory phosphorothioate oligonucleotide adjuvant. Vaccine 2003;21:2461–7.
[32] Galbraith WM, Hobson WC, Giclas PC, Schechter PJ, Agrawal S. Complement activation and hemodynamic changes following intravenous administration of phosphorothioate oligonucleotides in the monkey. Antisens Res Dev 1994;4:201–6.
[33] Brazolot Millan CL, Weeratna R, Krieg AM, Siegrist CA, Davis HL. CpG DNA can induce strong Th1 humoral and cell-mediated immune responses against hepatitis B surface antigen in young mice. Proc Natl Acad Sci USA 1998;95:15553–8.
[34] Dong L, Mori I, Hossain MJ, Liu B, Kimura Y. An immunostimulatory oligodeoxynucleotide containing a cytidine-guanosine motif protects senescence-accelerated mice from lethal influenza virus by augmenting the T helper type 1 response. J Gen Virol 2003;84:1623–8.
[35] Manning BM, Enioutina EY, Visic DM, Knudson AD, Daynes RA. CpG DNA functions as an effective adjuvant for the induction of immune responses in aged mice. Exp Gerontol 2001;37:107–26.
[36] Davis HL, Cooper CL, Morris ML, Elfer SM, Cameron DW, Heathcote J. CpG ODN is safe and highly effective in humans as adjuvant to HBV vaccine: preliminary results of phase I trial with CpG ODN 7909. In: 3rd Annual Conference on Vaccine Research; 2000. Washington, DC; 1 May 2000.
[37] Singh M, Ott G, Kazzaz J, Ugozzoli M, Briones M, Donnelly J, et al. Cationic microparticles are an effective delivery system for immune stimulatory cpG DNA. Pharm Res 2001;18:1476–9.
[38] Burton DR, Gregory L, Jefferis R. Aspects of the molecular structure of IgG subclasses. Monogr Allergy 1986;19:7–35.
[39] Spiegelberg HL. Biological activities of immunoglobulins of different classes and subclasses. Adv Immunol 1974;19:259–94.
[40] Murphy BR, Baron S, Chalhub EG, Uhlendorf CP, Chanock RM. Temperature-sensitive mutants of influenza virus. IV. Induction of interferon in the nasopharynx by wild-type and a temperature-sensitive recombinant virus. J Infect Dis 1973;128:488–93.
[41] Jao RL, Wheelock EF, Jackson GG. Production of interferon in volunteers infected with Asian influenza. J Infect Dis 1970;121:419–26.
[42] Hayden FG, Fritz R, Lobo MC, Alvord W, Strober W, Straus SE. Local and systemic cytokine responses during experimental human influenza A virus infection. Relation to symptom formation and host defense. J Clin Invest 1998;101:643–9.

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A phase I study of the safety and immunogenicity of recombinant hepatitis B surface antigen co-administered with an immunostimulatory phosphorothioate oligonucleotide adjuvant☆

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Abstract

Certain oligodeoxynuclotides with CpG motifs provide enhanced immune response to co-delivered antigens. We performed a phase I, observer-blinded, randomized study in healthy anti-hepatitis B surface antigen (anti-HBsAg) antibody negative adults to explore safety and immunogenicity of co-injection of recombinant HBsAg combined with an immunostimulatory DNA sequence (ISS) 1018 ISS. Four ISS dosage groups ($N = 12$ per group) were used: 300, 650, 1000 or 3000 μg. For each group, two controls received 20 μg HBsAg alone, two controls received ISS alone, and eight subjects received ISS + 20 μg HBsAg. Subjects received two doses 8 weeks apart. Injection site reactions (tenderness and pain on limb movement) were more frequent at higher ISS + HBsAg doses but were mainly mild and of short duration. Higher anti-HBsAg antibody levels were associated with higher ISS doses. Four weeks after the first dose, a seroprotective titer (≥10 mIU/ml) was noted for 0, 25, 75, and 87.5% of subjects by increasing ISS dose group ($P < 0.05$) for those who received ISS + HBsAg; 1 month after the second dose this increased to 62.5, 100, 100, and 100%, respectively. Geometric mean anti-HBsAg antibody levels by increasing ISS + HBsAg dose were 1.22, 5.78, 24.75, and 206.5 mIU/ml after the first dose and 65.37, 877.6, 1545, and 3045 mIU/ml after the second dose. We conclude that 1018 ISS + HBsAg was well tolerated and immunogenic in this phase I study in healthy adults and may offer the potential for enhancement of hepatitis B virus (HBV) immunization and protection after one or two doses or in individuals who fail to respond to the standard vaccine regimen.

Keywords: Hepatitis B vaccine; Immunostimulatory sequence; CpG motifs; Randomized controlled trial

1. Introduction

Hepatitis B virus (HBV) causes acute and chronic infection in humans and is responsible for substantial morbidity and mortality [1,2]. Although only 5–15% of acutely infected young children and 33–50% of older children and adults will manifest clinical illness, 5–10% of adults and as high as 90% of vertically infected infants will become chronically infected [3]. Chronically HBV infected individuals are at increased risk of subsequent development of hepatocellular carcinoma [4]. Worldwide, it is estimated that 5% of the world's population has chronic HBV infection and that 500,000 to 1 million people die annually from HBV-related liver disease [5]. Universal immunization against HBV is recommended throughout the world and is effective in preventing maternal to infant transmission and chronic infection, and in decreasing the incidence of hepatocellular carcinoma [6].

Currently available HBV vaccines in North America and Europe consist of recombinant hepatitis B surface antigen (HBsAg) adsorbed to aluminum hydroxide or aluminum phosphate. Protection against disease is associated with post-immunization antibody levels against HBsAg of ≥10 mIU/ml [7]. Protective levels are achieved in 90% or

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more healthy adults after a three-dose series of vaccine given at 0, 1, and 6 months although rates of seroprotection are lower with increasing age, obesity, and in those who smoke [8]. Although accelerated 3-month schedules (0, 1, and 2 months) are routinely used in some jurisdictions, higher antibody levels are achieved with longer intervals between the second and third injection [9]. Two-dose regimens with an interval of at least 4 months between doses have been evaluated for use in adolescents in whom the need for multiple doses may lead to decreased compliance with completion of the immunization series [10]. Similarly, in the developing world where hepatitis B remains a significant cause of morbidity and mortality, a single-dose regimen or an accelerated two-dose regimen would be useful to increase rates of HBV immunity [11]. A more immunogenic vaccine is also needed which would be effective in high-risk individuals who fail to respond to the standard immunization series.

Immunostimulatory DNA sequences (ISS) are emerging as useful tools for modulating immune responses. ISS are components of bacterial but not vertebrate DNA that have potent NK activation and interferon-inducing properties [12] which can be reproduced by certain synthetic oligonucleotides containing CpG motifs [13,14]. ISS stimulate the production of Th1-type cytokines such as IL-12 and interferons from a variety of cells such as dendritic cells, macrophages and NK cells [15–17]. ISS also stimulate B-cell proliferation and immunoglobulin secretion [18–20] as well as activation of antigen presenting cells [21,22]. ISS have potent Th1 adjuvant properties when used for immunization with DNA [23,24] or protein [17,25–27] vaccines. We studied the safety and immunogenicity of a 22-mer synthetic, phosphorothioate oligodeoxyribonuclotide immunostimulatory sequence (1018 ISS) co-administered with HBsAg in healthy adults.

2. Materials and methods

2.1. Vaccine

Study products consisted of 1018 ISS (sequence 5′-TGACTGTGAACGTTCGAGATGA-3′; Dynavax Technologies, Berkeley, CA) alone and in combination with yeast recombinant HBsAg without aluminum hydroxide adjuvant (Rhein Americana S.A., Buenos Aires, Argentina). Study products were stored at −60 °C or below and used within 8 h of thawing, and were diluted with sterile phosphate buffered saline to achieve the desired concentrations. Study vaccines were reconstituted by the study pharmacist to contain 300, 650, 1000, or 3000 μg of 1018 ISS alone or mixed with 20 μg HBsAg.

2.2. Populations

Healthy adults between 18 and 55 years of age were eligible if they had no history of hepatitis B infection or immunization with hepatitis B vaccine, had negative tests for HBsAg and antibodies against HBsAg and HBcAg. Individuals were excluded from enrollment if they were pregnant or unwilling to use effective contraception during the study, had clinically significant acute or chronic diseases, had any immunosuppressive disorders or medication, had prior injection of DNA plasmids or oligonuclotides, had behavioral risk factors that might have resulted in recent exposure to hepatitis B virus, had received blood products or immunoglobulin within 3 months of study entry, had a history of sensitivity to any component of the study vaccines or had abnormalities of screening blood chemistries, hematology, or urinalysis.

2.3. Study design and procedures

The study was designed as a single center, randomized, controlled, observer-blinded, dose escalating study in 48 healthy adult volunteers. Written, informed consent was obtained from all participants prior to any study procedure; the study was approved by the Research Ethics Board of the IWK Health Centre, Halifax, NS, Canada. The first 12 participants were randomly allocated in a ratio of 2:2:8 by computer generated list to receive either 20 μg HBsAg, 300 μg 1018 ISS, or 20 μg HBsAg + 300 μg 1018 ISS. Only the study pharmacist who mixed the vaccines at the time of immunization and who was not involved in any other aspects of the study was aware of the vaccine allocation. The second cohort of 12 participants was randomly allocated to receive 20 μg HBsAg, 650 μg ISS, or 20 μg HBsAg + 650 μg 1018 ISS in a ratio of 2:2:8. Dose escalation occurred after review by the investigator, sponsor, and an independent medical monitor of the safety results of the cohort who had received the previous dose level. The third and fourth cohorts of 12 subjects received 1000 or 3000 μg 1018 ISS with or without 20 μg HBsAg, respectively, in the same ratio.

Two doses of the same vaccine were given as intramuscular injections into opposite deltoid muscles 2 months apart. Participants were monitored by study personnel for 30 min after the immunization for any immediate adverse events and by the subjects themselves using a symptom diary for 7 days post-immunization. Participants were instructed to measure their temperature daily and to assess specific injection site adverse events (redness, swelling, warmth, tenderness, pain with arm movement) and systemic adverse events (chills, headache, muscle aches, fatigue, nausea, vomiting, diarrhea, joint pain); all reports of other adverse events were collected and categorized by body system. Serious adverse events were defined as events that were fatal or life-threatening; caused or prolonged hospitalization; resulted in a significant, persistent, or permanent disability; produced a congenital anomaly; or required intervention to prevent permanent impairment or damage. Solicited adverse events were either measured (fever, redness, swelling) or categorized as mild (awareness of symptom but easily tolerated),

moderate (discomfort enough to cause interference with usual activity), or severe (incapacitating with inability to work or do usual activity). Adverse events were collected by study personnel during visits 7 and 28 days after each immunization.

Blood was collected by venipuncture immediately before 1 week and 1 month after each dose of vaccine for measurement of serum biochemical and hematological parameters, complement (C3 and C4), erythrocyte sedimentation rate, and antinuclear and anti-single stranded and -double stranded DNA. Serum antibody response to HBsAg was measured by enzyme immunoassay (AUSAB EIA, Abbott Laboratories, Abbott Park); all testing was performed in a blinded fashion on code labeled, matched pre- and post-immunization sera. All anti-HBsAg antibody levels were expressed as mIU/ml. At the completion of the study, licensed hepatitis B vaccine was provided to participants who had not achieved antibody levels ≥100 mIU/ml.

2.4. Data analysis and statistical considerations

Adverse events were tabulated by time (day) and by severity (mild, moderate, and severe). The maximum size and severity was used within each time period. Clinically significant reactions were defined as measured reactions ≥10 mm, fever ≥38 °C, and severity ≥ moderate for other symptoms. Severe reactions were defined as measured reactions ≥50 mm, fever ≥39 °C, and severity "severe" for all other symptoms. Injection site reactions were combined to give an "any local" reaction category and all other reactions combined to give an "any general" reaction. The proportion of subjects having an adverse reaction was estimated by vaccine group, observation period and severity. Binomial distribution point estimates and 95% confidence intervals were used to estimate each rate; percentages were compared by Fisher's exact test. $P < 0.05$ was considered statistically significant; no adjustments were made for multiple comparisons.

Geometric mean antibody levels and 95% confidence intervals were estimated pre- and post-immunization and compared across groups by ANOVA; linear regression was used to assess trend across dose level. The proportion of each vaccine group achieving seroprotective antibody levels (≥10 mIU/ml) post-immunization was compared by Fisher's exact test.

The primary outcome of the study was the proportion of subjects reporting specific post-injection reactions. The secondary outcome was the proportion of subjects seroprotected after immunization and the geometric mean antibody level. As this was a phase I, first-in-human clinical trial, no formal hypothesis testing was planned and no formal sample size calculation was performed; however, each treatment group sample size (eight participants) ensured that the probability of detecting at least one adverse event in the group was 0.73, provided that the true adverse event rate exceeded 15%.

3. Results

3.1. Demographics

A total of 74 subjects provided written, informed consent and underwent pre-study screening; reasons for "screen failures" included persistently abnormal baseline biochemistry or hematology tests [14], pre-existing antibody against hepatitis B virus [4], hypertension [1], inability to contact after the screening visit [2], withdrawal of consent [1], target enrollment reached before subject could be scheduled [4]. The remaining 48 participants were randomized and received study drug; all but one completed the study. The mean age of participants was 33 years (range 18–52 years) and 63% were women. There were no differences in the age or gender distribution amongst the vaccine groups. All but one participant completed participation in the study; one subject withdrew consent because of adverse events after the first injection.

3.2. Adverse events

3.2.1. Clinical adverse events

No adverse events were reported in the first 30 min after injection. During the first 7 days after each dose, solicited adverse events were common; an adverse event was reported after the first dose by 2 (25%) HBsAg recipients, 6 (75%) of the combined ISS recipients, and 5 (62.5%) of the 300 μg ISS+HBsAg recipients, and 7 (87.5%) of each of the 650 μg, 1000 μg, and 3000 μg ISS+HBsAg recipients ($P = 0.04$ for the comparison of the latter 3 with HBsAg). After the second dose, solicited adverse events of any type were reported by 5 (62.5%), 5 (62.5%), 3 (37.5%), 1 (12.5%), 5 (71.4%) and 5 (62.5%) (P not significant for all comparisons). Both local and systemic adverse events were reported (Table 1). Injection site adverse events were for the most part reported within the first 24 h after the injection whereas the more non-specific systemic adverse events (such as headache, diarrhea) were reported throughout the first 7 days after the injection (data not shown). Mild tenderness at the injection site and mild pain on motion of the injected limb were the most commonly reported adverse events and were significantly more frequent in the 1000 μg and 3000 μg ISS + HBsAg groups for tenderness and the 3000 μg + HBsAg group for motion pain; these events were short and self limited and did not require any medical treatment or intervention. There was a significant ISS dose-related trend for increased tenderness ($P = 0.01$) and pain on limb motion ($P = 0.05$). Warmth at the injection site was only reported by three participants (one each in the HBsAg alone, 300 μg ISS+HBsAg and 1000 μg ISS + HBsAg groups). Only one severe (≥50 mm) injection site adverse event (redness) was reported; redness of 50 mm was reported after the first dose of 1000 μg ISS + HBsAg and resolved with 24 h without treatment. Injection site adverse events did not increase with the second dose compared to the first.

Table 1
Adverse events reported within 7 days after each of two doses of HBsAg and/or 1018 ISS and/or HBsAg combined with varying quantities of 1018 ISS in 48 healthy adult volunteers

Adverse event	Dose	Severity	Vaccine number (%) reporting adverse event					
			ISS[a] ($N = 8$)	HBsAg ($N = 8$)	HBsAg + 300 μg ISS ($N = 8$)	HBsAg + 650 μg ISS ($N = 8$)	HBsAg + 1000 μg ISS ($N = 8$)[b]	HBsAg + 3000 μg ISS ($N = 8$)
Redness	1	Any	0	0	0	0	1 (12.5)	1 (12.5)
		Moderate/severe	0	0	0	0	1 (12.5)	1 (12.5)
	2	Any	0	1 (12.5)	1 (12.5)	0	2 (28.6)	0
		Moderate/severe	0	1 (12.5)	1 (12.5)	0	1 (14.3)	0
Swelling	1	Any	0	0	1 (12.5)	1 (12.5)	1 (12.5)	0
		Moderate/severe	0	0	1 (12.5)	1 (12.5)	1 (12.5)	0
	2	Any	0	1 (12.5)	0	0	1 (14.3)	0
		Moderate/severe	0	0	0	0	0	0
Tenderness	1	Any	3 (37.5)	0	2 (25)	1 (12.5)	6 (75)[c]	5 (62.5)[d]
		Moderate/severe	1 (12.5)	0	0	0	0	1 (12.5)
	2	Any	1 (12.5)	2 (25)	1 (12.5)	0	4 (57.1)	5 (62.5)
		Moderate/severe	0	1 (12.5)	0	0	1 (14.3)	0
Pain on movement	1	Any	4 (50)	1 (12.5)	2 (25)	3 (37.5)	5 (62.5)	6 (75)[e]
		Moderate/severe	0	0	0	0	0	0
	2	Any	3 (37.5)	1 (12.5)	0	1 (12.5)	3 (42.9)	4 (50)
		Moderate/severe	0	0	0	0	2 (28.6)	1 (12.5)
Muscle aches	1	Any	0	2 (25)	1 (12.5)	2 (25)	1 (12.5)	3 (37.5)
		Moderate/severe	0	1 (12.5)	0	0	1 (12.5)	0
	2	Any	0	2 (25)	0	0	0	1 (12.5)
		Moderate/severe	0	0	0	0	0	0
Headache	1	Any	1 (12.5)	1 (12.5)	3 (37.5)	4 (50)	5 (62.5)	1 (12.5)
		Moderate/severe	0	0	2 (25)	0	2 (25)	0
	2	Any	3 (37.5)	3 (37.5)	3 (37.5)	0	2 (28.6)	0
		Moderate/severe	0	2 (25)	3 (37.5)	0	2 (28.6)	0
Fatigue	1	Any	0	1 (12.5)	0	3 (37.5)	4 (50)	2 (25)
		Moderate/severe	0	0	0	1 (12.5)	1 (12.5)	1 (12.5)
	2	Any	0	1 (12.5)	0	0	1 (14.3)	0
		Moderate/severe	0	0	0	0	1 (14.3)	0
Nausea	1	Any	0	0	0	0	3 (37.5)	1 (12.5)
		Moderate/severe	0	0	0	0	1 (12.5)	1 (12.5)
	2	Any	1 (12.5)	0	1 (12.5)	0	2 (28.6)	1 (12.5)
		Moderate/severe	0	0	0	0	0	0
Joint pain	1	Any	0	1 (12.5)	1 (12.5)	0	0	1 (12.5)
		Moderate/severe	0	0	0	0	0	0
	2	Any	0	2 (25)	0	0	1 (14.3)	1 (12.5)
		Moderate/severe	0	0	0	0	0	0

[a] Includes all participants who received ISS alone (two volunteers per ISS concentration).
[b] Only seven participants in this group received the second injection.
[c] $P < 0.001$ for comparison with HBsAg.
[d] $P = 0.03$ for comparison with HBsAg.
[e] $P = 0.04$ for comparison with HBsAg.

Headache was the most commonly reported solicited systemic adverse event but was not associated with any particular study product or dose (Table 1). Fatigue and muscle aches were also commonly reported but again there were no differences between the study groups or doses. Fever (38.9 °C) was reported by one recipient of HBsAg alone with onset >72 h after the second injection; there were no reports of vomiting. Chills (all mild) were reported by one recipient of the 650 μg ISS + HBsAg, 1000 μg ISS + HBsAg, and two recipients of HbsAg (both >24 h after the second dose); diarrhea (none severe) was reported by one each recipient of ISS alone, 300 μg ISS + HBsAg, 1000 μg ISS + HBsAg and two recipients of HbsAg alone; three of the five were reported as moderate and all had onset >24 h after the injection. Three of the systemic adverse events were described as severe including nausea with onset >72 h after the first dose in a recipient of 1000 μg ISS + HBsAg, headache with onset >72 h after the first dose in a recipient of 300 μg ISS + HBsAg, and fatigue with onset >24 h after the first dose in a recipient of 650 μg ISS + HBsAg. All systemic adverse events

resolved within 24 h and none required treatment of medical intervention. There were no differences amongst the groups in unsolicited adverse events.

There were no serious adverse events during the study. One subject withdrew consent after reporting injection site swelling and erythema and moderate disorientation, dizziness, myalgia, and fatigue and mild shortness of breath several hours after the first injection with 1000 μg ISS+HBsAg; all symptoms resolved within 72 h of the injection.

3.2.2. Laboratory abnormalities

Mild abnormalities in clinical chemistries and hematology values were demonstrated in participants during the study; however, there was no discernable pattern or association with a specific study product. Immediately prior to the second dose (56 days after the first dose), 5 (62.5%) of 8 participants in the 3000 μg ISS + HBsAg group had mildly elevated ALT (mean 42.5 u/ml, maximum 72 u/ml; laboratory normal range 5–35 u/ml for females, 10–40 u/ml for males) and AST levels (mean 36.5 u/ml, maximum 61 u/ml; laboratory normal range 5–35 u/ml for females, 10–45 u/ml for males); of these, 3 (37.5%) and 2 (25%) were still mildly elevated 1 week after the second dose. Changes in complement levels or development of antinuclear or anti-DNA antibodies were not observed during the study.

3.3. Antibody response

At baseline, all participants were seronegative for anti-HBsAg antibodies (Table 2). No participants who were immunized with ISS alone developed antibodies against HBsAg. A total of 2 (25%) of the 8 recipients of HBsAg alone (without ISS) developed measurable antibodies (seroresponded) against HBsAg which were only detectable 4 months post-dose 2; neither participant was seroprotected (titer <10 mIU/ml).

All but one participant immunized with ISS + HBsAg responded to HBsAg with measurable antibody levels (a participant immunized with 300 μg ISS + HBsAg). All but two were seroprotected (the 300 μg ISS + HBsAg recipient and a 1000 μg ISS + HBsAg recipient who withdrew from the study after a single dose of vaccine). A statistically significant dose-related trend was demonstrated for both amplitude of the immune response and the rapidity of achieving an immune response (geometric mean titer; $P < 0.001$). At the highest dose level administered (3000 μg ISS + HBsAg), a geometric mean titer of 206.5 mIU/ml was achieved 28 days after the first dose and 1429 and 3045 mIU/ml 7 and 28 days, respectively, after dose 2. At this dose level, 3 (37.5%) of 8 participants were seroprotected 7 days after the first dose which increased to 7 (87.5%) of 8 by 28 days post-dose 1; all participants in the group were seroprotected 7 days after the second dose (Fig. 1). At the next lower dose of ISS + HBsAg (1000 μg), protective responses were not detected in the 7-day post-dose 1 sample but six of the eight participants achieved protective levels by 28 days after the first dose and all seven participants were seroprotected prior to the second dose (the eighth participant withdrew from the study before receiving the second dose). With the two lowest doses of ISS, the majority of participants required a second dose to achieve protective levels.

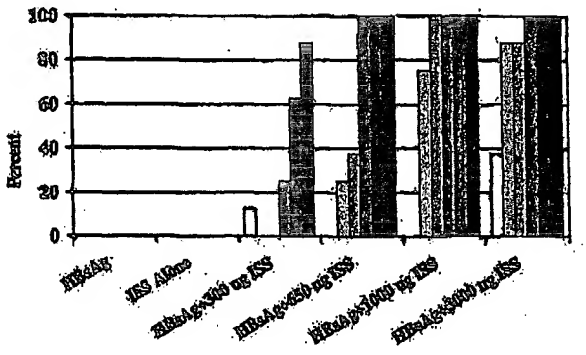

Fig. 1. Proportion of participants achieving a protective antibody level (≥10 mIU/ml) at various time points after immunization with study vaccines. Time points are (by increasing darkness of bar shade) 7 days after dose 1, 28 days after dose 1, 56 days after dose 1 (immediately prior to dose 2), 7 days after dose 2, 28 days after dose 2, 4 months after dose 2.

Table 2
Geometric mean anti-HBsAg antibody titer after each of two doses of HBsAg and/or 1018 ISS and/or HBsAg combined with varying quantities of 1018 ISS in 48 healthy adult volunteers

Time post-immunization	Vaccine geometric mean antibody titer (95% confidence interval)					
	ISS ($N = 8$)	HBsAg ($N = 8$)	HBsAg + 300 μg ISS ($N = 8$)	HBsAg + 650 μg ISS ($N = 8$)	HBsAg + 1000 μg ISS ($N = 8$)	HBsAg + 3000 μg ISS ($N = 8$)
Baseline (pre-dose 1)	1.00 (1.0–1.0)	1.00 (1.0–1.0)	1.00 (1.0,1.0)	1.00 (1.0–1.0)	1.00 (1.0–1.0)	1.00 (1.0–1.0)
7 days post-dose 1	1.00 (1.0–1.0)	1.00 (1.0–1.0)	1.44 (0.61–3.37)	1.00 (1.0–1.0)	1.00 (1.0–1.0)	10.76 (0.53–219.2)[a]
28 days post-dose 1	1.00 (1.0–1.0)	1.00 (1.0–1.0)	1.22 (0.76–1.97)	5.78 (1.72–19.43)	24.75 (4.7–130.4)[a]	206.5 (13.49–3160)[a]
Pre-dose 2 (56 days post-dose 1)	1.00 (1.0–1.0)	1.00 (1.0–1.0)	1.19 (0.79–1.79)	4.27 (1.18–15.47)[b]	46.59 (23.34–93.01)[a]	84.42 (9.87–722.1)[a]
7 days post-dose 2	1.00 (1.0–1.0)	1.00 (1.0–1.0)	2.83 (0.89–9.01)	82.37 (27.45–247.2)[a]	316.1 (133.5–748.8)[a]	1429 (256.8–7954)[a]
28 days post-dose 2	1.00 (1.0–1.0)	1.00 (1.0–1.0)	65.37 (6.63–644.5)[a]	877.6 (326.0–2362)[a]	1545 (689.3–3463)[a]	3045 (641.8–14446)[a]
4 months post-dose 2	1.00 (1.0–1.0)	1.32 (0.86–2.01)	83.24 (16.18–428.2)[a]	756.0 (253.2–2257)[a]	1713 (699.5–4196)[a]	1206 (244.8–5939)[a]

[a] $P < 0.001$ for comparison with ISS alone or HBsAg.
[b] $P = 0.04$ for comparison with ISS alone or HBsAg.

4. Discussion

The results of this phase I study indicate that all dose levels of 1018 ISS+HBsAg vaccine were well tolerated (except in one individual who withdrew consent because of the local and systemic adverse events) and immunogenic in these healthy adults. Although all four ISS + HBsAg doses tested were more immunogenic than HBsAg alone, the 1000 and 3000 μg doses induced rapid and high antibody levels after one or two injections. Injection site adverse events were mostly mild, of short duration, and self-limited, and tended to increase with increasing dose of 1018 ISS; however, adverse events did not increase in frequency with the second dose. Injection site adverse events were reported more frequently in participants who received 1018 ISS compared to those immunized with HBsAg alone whereas systemic adverse events were reported with similar frequency amongst all study participants. Pain at the injection site and limitation of limb motion were the most frequent events, reported in as many as 50–67.5% of participants. Although these rates may appear high, in part this may be a result of the active surveillance involved in a phase I study. Concurrent comparison with a licensed HBV (containing adjuvant) would be informative as these vaccines have an acceptable injection site adverse event profile under routine use. The rates of injection site adverse events in this study were similar to those reported by other investigators with licensed, alum adjuvanted HBV [28] and from this center with a licensed diphtheria–tetanus toxoid vaccine studied using similar surveillance methods [29].

Although a concurrent licensed HBV control would have been ideal, comparison of the antibody responses achieved in this study to those reported with licensed vaccines is less problematic. In this study, 13 (81%) of 16 participants who received 1000 or 3000 μg 1018 ISS demonstrated protective levels of anti-HBsAg antibody 28 days after the first injection and 100% seroprotection after the second dose, using an immunization interval of 2 months. By comparison, licensed HBV is reported to elicit a seroprotective response in up to 20% of healthy young adults after the first dose and up to 71% of recipients 1 month after the second dose with a less effective 1 month interval [28,30–34]. Geometric mean antibody titers with licensed HBV given on a 0, 6 months schedule were reported as 1203 mIU/ml 1 month after the second injection [34] compared to 1545 and 3045 mIU/ml for 1 month after the second 1000 and 3000 μg 1018 ISS doses, respectively, in this study. Geometric mean antibody titers 1 month after a single dose of 3000 μg ISS + HBsAg were 206 mIU/ml compared to 5–27 mIU/ml 1 month after a second injection on a 0, 1 month schedule with licensed HBV [31,35]. High anti-HBsAg antibody titers were maintained 4 months after the second dose of ISS + HBsAg with no decrease in antibody levels in recipients of 300, 650, or 1000 μg ISS + HBsAg and levels still exceeding 1000 mIU/ml in recipients of 3000 μg ISS + HBsAg, despite some decrease in GMT.

Pre-clinical studies demonstrated activity of 1018 ISS in mice, rabbits, dogs, and non-human primates (baboons, cynomolgus monkeys) with induction of a Th1 type of cytokine response in mice [36]. In primate studies with 1018 ISS, similar rates of seroprotection were achieved with a single dose of 1018 ISS + HBsAg [36]. Although in a preliminary communication enhanced immunogenicity of another CpG co-administered with alum-adsorbed HBV has been reported in humans [37], this is the first report in humans using CpG as the sole adjuvant with HBsAg.

The accelerated antibody response and increase antibody titers elicited after one or two doses indicate that the 1018 ISS + HBsAg vaccine may be useful in immunizing difficult to access populations such as adolescents, individuals with high-risk behaviors that increase their risk of hepatitis B, health care workers, and children in developing nations. Other potential uses for a hepatitis B vaccine with enhanced immunogenicity would be populations known to be hypo- or non-responders such as older individuals, renal dialysis patients, and immune compromised hosts [38–41]. The results of this phase I study support further studies with 1018 ISS+HBsAg in healthy adults and hypo- and non-responders to licensed hepatitis B vaccines.

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References

[1] Centers for Disease Control and Prevention. Hepatitis B vaccination—United States, 1982–2002. MMWR Morb Mortal Wkly Rep 2002;51:549–63.
[2] Lee WM. Hepatitis B virus infection. N Engl J Med 1997;337:1733–43.
[3] McMahon BJ, Alward WL, Hall DB, Heyward WL, Bender TR, Francis DP, et al. Acute hepatitis B virus infection: relation of age to the clinical expression of disease and subsequent development of the carrier state. J Infect Dis 1985;151:599–603.
[4] Beasley RP, Hwang L-Y. Overview on the epidemiology of hepatocellular carcinoma. In: Hollinger FB, Lemon SB, Margolis HS, editors. Viral hepatitis and liver disease. Baltimore: Williams & Wilkins; 1991. p. 532–5.
[5] Maynard JE, Kane MA, Alter MJ, Halder SC. Control of hepatitis B by immunization: global perspectives. In: Vyas GN, Dienstag JL, Hoofnagle JH, editors. Viral hepatitis and liver disease. New York: Grune & Stratton; 1988. p. 967–9.
[6] Huang K, Lin S. Nationwide vaccination: a success story in Taiwan. Vaccine 2000;18(Suppl 1):S35–8.
[7] Szmuness W, Stevens CE, Harley EJ, Zang EA, Oleszko WR, William DC, et al. Hepatitis B vaccine: demonstration of efficacy

in a controlled trial in a high risk population in the U.S. N Engl J Med 1980;303:833–41.
[8] Andre FE. Summary of safety and efficacy data on a yeast-derived hepatitis B vaccine. Am J Med 1989;87(Suppl 3a):39–45.
[9] Mahoney FJ, Kane M. Hepatitis B vaccine. In: Plotkin SA, Orenstein WA, editors. Vaccines. Philadelphia: Saunders; 1999. p. 158–82.
[10] Cassidy WM, Watson B, Ioli VA, Williams K, Bird S, West DJ. A randomized trial of alternative two- and three-dose hepatitis B vaccination regimens in adolescents: antibody responses, safety, and immunologic memory. Pediatrics 2001;107:626–31.
[11] Centers for Disease Control and Prevention. Hepatitis B vaccination among high-risk adolescents and adults—San Diego, California, 1998–2001. MMWR Morb Mortal Wkly Rep 2002;51: 618–21.
[12] Yamamoto S, Yamamoto T, Shimada S, Kuramoto E, Yano O, Kataoka T, et al. DNA from bacteria, but not from vertebrates, induces interferons, activates natural killer cells and inhibits tumor growth. Microbiol Immunol 1992;36:983–7.
[13] Yamamoto S, Yamamoto T, Kataoka T, Kuramoto E, Yano O, Tokunaga T. Unique palindromic sequences in synthetic oligonucleotides are required to induce IFN and augment IFN-mediated natural killer activity. J Immunol 1992;148:4072–6.
[14] Sonehara K, Saito H, Kuramoto E, Yamamoto S, Yamamoto T, Tokunaga T. Hexamer palindromic oligonucleotides with 5′-CG-3′ motif(s) induce production of interferon. J Interferon Cytokine Res 1996;16:799–803.
[15] Halpern MD, Kurlander RJ, Pisetsky DS. Bacterial DNA induces murine interferon-gamma production by stimulation of interleukin-12 and tumor necrosis factor-alpha. Cell Immunol 1996;167:72–8.
[16] Klinman DM, Yi AK, Beaucage SL, Conover J, Krieg AM. CpG motifs present in bacteria DNA rapidly induce lymphocytes to secrete interleukin 6, interleukin 12, and interferon gamma. Proc Natl Acad Sci USA 1996;93:2879–83.
[17] Roman M, Martin-Orozco E, Goodman JS, Nguyen MD, Sato Y, Ronaghy A, et al. Immunostimulatory DNA sequences function as T helper-1-promoting adjuvants. Nat Med 1997;3:849–54.
[18] Krieg AM, Matson S, Fisher E. Oligodeoxynucleotide modifications determine the magnitude of B cell stimulation by CpG motifs. Antisense Nucelic Acid Drug Dev 1996;6:133–9.
[19] Krieg AM, Yi AK, Matson S, Waldschmidt TJ, Bishop GA, Teasdale R, et al. CpG motifs in bacterial DNA trigger direct B-cell activation. Nature 1995;374:546–9.
[20] Liang H, Nishioka Y, Reich CF, Pisetsky DS, Lipsky PE. Activation of human B cells by phosphorothioate oligodeoxynucleotides. J Clin Invest 1996;98:1119–29.
[21] Hartmann G, Weiner GJ, Krieg AM. CpG DNA: a potent signal for growth, activation, and maturation of human dendritic cells. Proc Natl Acad Sci USA 1999;96:9305–10.
[22] Martin-Orozco E, Kobayashi H, Van Uden J, Nguyen MD, Kornbluth RS, Raz E. Enhancement of antigen-presenting cell surface molecules involved in cognate interactions by immunostimulatory DNA sequences. Int Immunol 1999;11:1111–8.
[23] Klinman DM, Yamshchikov G, Ishigatsubo Y. Immunostimulatory DNA sequences necessary for effective intradermal gene immunization. J Immunol 1997;158:3635–9.
[24] Sato Y, Roman M, Tighe H, Lee D, Corr M, Nguyen MD, et al. Immunostimulatory DNA sequences necessary for effective intradermal gene immunization. Science 1996;273:352–4.
[25] Weiner GJ, Liu HM, Wooldridge JE, Dahle CE, Krieg AM. Immunostimulatory oligodeoxynucleotides containing the CpG motif are effective as immune adjuvants in tumor antigen immunization. Proc Natl Acad Sci USA 1997;94:10833–7.
[26] Chu RS, Targoni OS, Krieg AM, Lehmann PV, Harding CV. CpG oligodeoxynucleotides act as adjuvants that switch on T helper 1 (Th1) immunity. J Exp Med 1997;186:1623–31.
[27] Davis HL, Weeranta R, Waldschmidt TJ, Tygrett L, Schorr J, Krieg AM. CpG DNA is a potent enhancer of specific immunity in mice immunized with recombinant hepatitis B surface antigen. J Immunol 1998;160:870–6.
[28] Heineman T, Clements-Mann ML, Poland GA, Jacobson RM, Izu AE, Sekamoto D, et al. A randomized, controlled study in adults of the immunogenicity of a novel hepatitis B vaccine containing MF59 adjuvant. Vaccine 1999;17:2769–78.
[29] Halperin SA, Smith B, Russell M, Hasselback P, Guasparini R, Skowronski D, et al. An adult formulation of a five-component acellular pertussis vaccine combined with diphtheria and tetanus toxoids is safe and immunogenic in adolescents and adults. Vaccine 2000;18:1312–9.
[30] Andre F, Safary A. Summary of clinical findings on Engerix-B a genetically engineered yeast-driven hepatitis B vaccine. Postgrad Med J 1987;63:169–78.
[31] Miskovsky E, Gershman K, Clements ML, Cupps T, Calandra G, Hesley T, Ioli V, et al. Comparative safety and immunogenicity of yeast recombinant hepatitis B vaccines containing S and pre-S2 + S. Vaccine 1991;9:346–50.
[32] Bryan JP, Craig PG, Reyes L, Hakre S, Jaramillo R, Harlan H, et al. Randomized comparison of 5 and 10 μg doses of two recombinant hepatitis B vaccines. Vaccine 1995;13:978–82.
[33] Jilg W, Schmidt M, Deinhardt F. Vaccination against hepatitis B: comparison of three different vaccination schedules. J Infect Dis 1989;160:766–9.
[34] Ambrosch F, Wiedermann G, Kundi M, Leroux-Roels G, Desombere I, Garcon N, et al. A hepatitis B vaccine formulated with a novel adjuvant system. Vaccine 2000;18:2095–101.
[35] Cassidy WM, Watson B, Ioli VA, Williams K, Bird S, West DJ. A randomized trial of alternative two- and three-dose hepatitis B vaccination regimens in adolescents: antibody responses, safety, and immunological memory. Pediatrics 2001;107:626–31.
[36] Van Nest G, Tighe H, Raz E, Higgins D, Traquina P, Eiden J. An immunostimulatory oligonucleotide (ISS ODN) enhances immune response to HBV vaccine in a variety of animal species including primates. In: Proceedings of the 39th Interscience Conference on Antimicrobial Agents and Chemotherapy, San Francisco, 1999 [Abstract 374].
[37] Davis H, Cooper CL, Morris ML, Elfer SM, Cameron DW, Heathcote J. CpG ODN is safe and highly effective in humans as adjuvant to HBV vaccine: preliminary results of phase I trial with CpG ODN 7909. In: Proceedings of the Third Annual Conference on Vaccine Research, Washington, DC, May 2000 [Abstract S25].
[38] Denis F, Mounier M, Hessel L, Michel JP, Gualde N, Dubois F, et al. Hepatitis B vaccination in the elderly. J Infect Dis 1984;149:1019.
[39] Heyward WL, Bender TR, McMahon BJ, Hall DB, Francis DP, Lanier AP, et al. The control of hepatitis B virus infection with vaccine in Yupik Eskimos. Am J Epidemiol 1995;121:914–23.
[40] McLean AA, Hilleman MR, McAleer WJ, Buynak EB. Summary of worldwide experience with HB-Vx (R) (B, MSD). J Infect Dis 1983;7:95–104.
[41] Weber DJ, Rutala WA, Samsa GP, Santimaw JE, Lemon SM. Obesity as a predictor of poor antibody response to hepatitis B plasma vaccine. J Am Med Assoc 1985;254:3187–9.

Rapid and strong human CD8+ T cell responses to vaccination with peptide, IFA, and CpG oligodeoxynucleotide 7909

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The induction of potent CD8+ T cell responses by vaccines to fight microbes or tumors remains a major challenge, as many candidates for human vaccines have proved to be poorly immunogenic. Deoxycytidyl-deoxyguanosin oligodeoxynucleotides (CpG ODNs) trigger Toll-like receptor 9, resulting in dendritic cell maturation that can enhance immunogenicity of peptide-based vaccines in mice. We tested whether a synthetic ODN, CpG 7909, could improve human tumor antigen–specific CD8+ T cell responses. Eight HLA-A2+ melanoma patients received 4 monthly vaccinations of low-dose CpG 7909 mixed with melanoma antigen A (Melan-A; identical to MART-1) analog peptide and incomplete Freund's adjuvant. All patients exhibited rapid and strong antigen-specific T cell responses: the frequency of Melan-A–specific T cells reached over 3% of circulating CD8+ T cells. This was one order of magnitude higher than the frequency seen in 8 control patients treated similarly but without CpG and 1–3 orders of magnitude higher than that seen in previous studies with synthetic vaccines. The enhanced T cell populations consisted primarily of effector memory cells, which in part secreted IFN-γ and expressed granzyme B and perforin ex vivo. In vitro, T cell clones recognized and killed melanoma cells in an antigen-specific manner. Thus, CpG 7909 is an efficient vaccine adjuvant that promotes strong antigen-specific CD8+ T cell responses in humans.

Introduction

A major goal of therapeutic cancer vaccines is the induction of large numbers of antigen-specific T cell populations with effector functions that are able to mediate immune protection. In contrast to viruses and other pathogens, vaccines containing recombinant proteins or synthetic antigenic peptides usually fail to induce significant immune responses unless they are mixed with adjuvants (1, 2). Effective adjuvants display at least 2 mechanisms of action: a depot effect that leads to prolonged antigen exposure in the host, and a capacity to trigger the innate immune system through activation of DCs via Toll-like receptors (TLRs) (3–5). Upon proper antigen presentation, activated DCs play a key role in the induction of T cell responses (6). Because of their high efficacy, several recently identified TLR ligands are promising vaccine adjuvants.

Synthetic deoxycytidyl-deoxyguanosin oligodeoxynucleotides (CpG ODNs) contain unmethylated CG motifs similar to those observed in bacterial DNA. CpG ODNs elicit a complex immunomodulatory cascade that includes the production of T helper-1–type cells and proinflammatory cytokines (7). CpG ODNs directly stimulate DC activation through TLR9 triggering (8, 9), leading to enhanced T cell responses specific for coadministered antigens in mice (10–14). For example, we have reported previously that addition of CpG ODNs to melanoma antigen A_{26-35} peptide (Melan-A_{26-35} peptide; a widely used antigenic peptide in vaccine trials of HLA-A2+ melanoma patients) mixed with incomplete Freund's adjuvant (IFA) increased Melan-A–specific T cell responses in HLA-A2 transgenic mice (15). However, the CpG motifs that stimulate the murine immune system are suboptimal for stimulating the human one. Indeed, TLR9-expressing human cells are susceptible to distinct CpG motifs (16–18). The recently described CpG 7909 has been optimized to stimulate human plasmacytoid DCs (pDCs) and B cells in vitro and in vivo (18).

Clinical studies have shown that CpG 7909 is a potent inducer of human innate immune responses and exhibits a strong adjuvant effect when coadministered with vaccines eliciting B cell responses against hepatitis B virus (19, 20). In contrast, it remains to be determined whether CpG ODNs are efficient adjuvants for vaccine-induced human cytolytic T cell responses (7). Given the well-documented but still relatively weak antigen-specific CD8+ T cell responses observed recently in melanoma patients vaccinated with Melan-A_{26-35} peptide and IFA (21–23), we tested whether coadministration of CpG 7909 to the same vaccine would enhance T cell responses. We have therefore performed a phase I clinical trial to examine toxicity and immunogenicity of this approach. Our results show rapid and consistent T cell responses in vivo, highlighting the potential of CpG 7909 to enhance cellular immune responses in humans.

Results

Vaccination with CpG 7909, peptide, and IFA caused no major side effects. Eight HLA-A2+ patients with advanced melanoma disease received 4 monthly subcutaneous injections of low doses of CpG 7909, Melan-A analog peptide, and IFA. Vaccination with this novel

Nonstandard abbreviations used: CpG, deoxycytidyl-deoxyguanosin; IFA, incomplete Freund's adjuvant; Melan-A, melanoma antigen A; ODN, oligodeoxynucleotide; pDC, plasmacytoid DC; TLR, Toll-like receptor.

Conflict of interest: A.M. Krieg is an employee of Coley Pharmaceutical Group Inc. and owns stock in this company. The other authors have declared that no conflict of interest exists.

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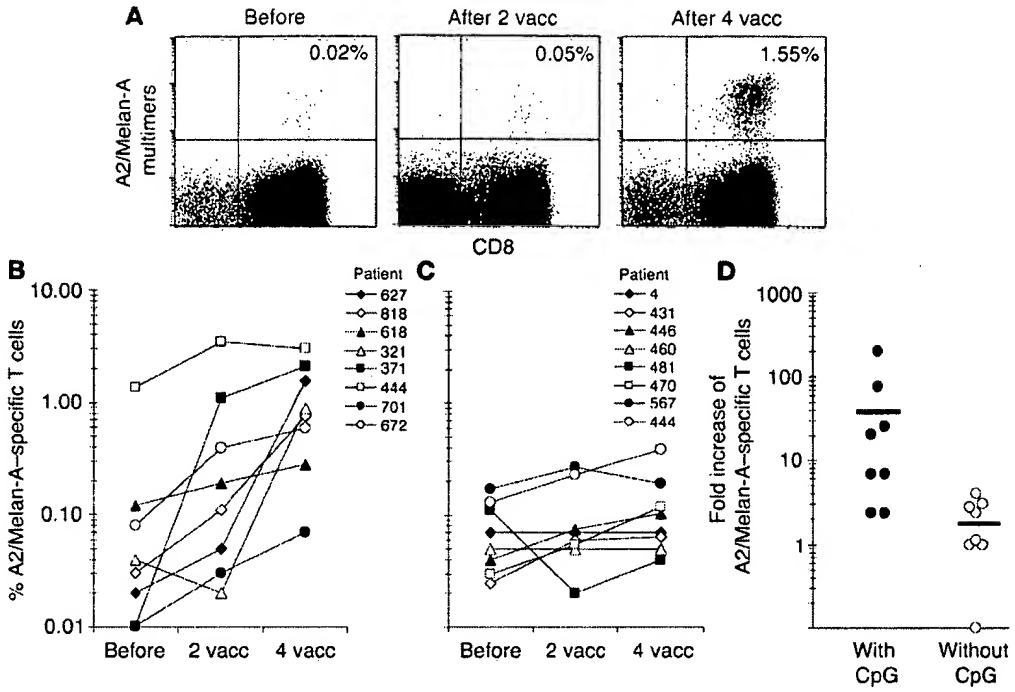

Figure 1

Rapid in vivo responses of Melan-A–specific T cells to vaccination (vacc) with low doses of CpG 7909, Melan-A peptide, and IFA. PBMCs were collected before, as well as 7–10 days after, vaccinations 2 and 4, and they were analyzed ex vivo by flow cytometry. (**A**) Dot plots from PBMCs of patient LAU 627, with percentage of Melan-A–specific cells among $CD8^+$ T cells. (**B**) After 2 and 4 vaccinations, 6/8 and 8/8 patients, respectively, had significantly increased percentages (i.e., greater than 2-fold) of Melan-A–specific T cells. (**C**) A control group of 8 patients was similarly treated with Melan-A peptide and IFA but without CpG (23). After 2 vaccinations, none of the patients had more than 2-fold increased percentages. After 4 vaccinations, 4/8 patients had more than 2-fold increased frequencies, but percentages of Melan-A–specific T cells remained significantly ($P < 0.01$) lower as compared to those of CpG-vaccinated patients. (**D**) Fold increase of Melan-A–specific T cells before or after 4 vaccinations in patients vaccinated with or without CpG. Horizontal lines indicate mean values.

3-component vaccine caused no major side effects. Minor systemic side effects were transient and included myalgia (4 patients), arthralgia and fatigue (3 patients), and nausea, malaise, and headache (2 patients). Interestingly, all 8 patients developed inflammatory signs at subcutaneous injection sites, with a peak of symptoms (induration, erythema, mild to moderate pain) around 2 weeks after injection. In response to recall vaccinations at distant sites in another limb, 4 patients showed reactivation of previous injection sites by redeveloping local inflammatory signs. Histological examination of a biopsy of one such distant reactivation site showed nonspecific inflammation with predominant perivascular lymphocyte infiltration (data not shown).

Rapid and consistent in vivo generation of Melan-A–specific $CD8^+$ T cells. PBMCs collected before and after vaccination were analyzed ex vivo by flow cytometry using fluorescent CD8-specific antibody and HLA-A2/Melan-A peptide multimers. After 4 vaccinations with CpG 7909, Melan-A peptide, and IFA, all 8 patients exhibited increased frequencies of Melan-A–specific $CD8^+$ T cells (0.07–3.00%), resulting in significantly ($P < 0.01$) higher percentages than before vaccination (Figure 1, A and B).

After 4 monthly vaccinations, the 8 patients exhibited a frequency of 1.15% ± 0.93% (mean ± SD) Melan-A–specific $CD8^+$ T cells. A control group of 8 melanoma patients was treated similarly but without CpG (Figure 1C), resulting in significantly ($P < 0.01$) lower T cell frequencies (0.13% ± 0.11%). Also, the response rate was far below 100%, since only 4 out of 8 patients generated ex vivo detectable T cell responses after 4 vaccinations. CpG-vaccinated patients reached a mean of 43-fold higher Melan-A–specific $CD8^+$ T cell frequencies than before vaccination, whereas patients vaccinated without CpG reached a mean of 1.9-fold higher frequencies than before vaccination (Figure 1D).

The majority of T cell responses developed rapidly: 6 out of 8 patients had increased frequencies of Melan-A–specific $CD8^+$ T cells after just 2 vaccinations (Figure 1B). This is again in sharp contrast to previous results. In the control group (Figure 1C), the lack of early responses (after 2 vaccinations) and the relatively low response rate are characteristic of all other studies with low-dose synthetic vaccines (21–25). Interestingly, patient LAU 444, who already had high levels of Melan-A–specific $CD8^+$ T cells before vaccination (resulting from previous immunotherapy and a natural response to melanoma [26]), was the only patient whose frequency peaked after just 2 vaccinations. In the other 7 patients, maximal frequencies were reached after 4 vaccinations. To our knowledge, these results demonstrate for the first time in humans that a synthetic peptide-based vaccine, when coadministered with adequate adjuvant, can rapidly elicit ex vivo detectable $CD8^+$ T cell responses.

Two of the 8 patients (LAU 371 and LAU 321) had previously been unsuccessfully vaccinated with Melan-A peptide mixed with the immunological adjuvants MPL and QS21 (Figure 2). During 4–5 years, the frequencies of Melan-A–specific T cells

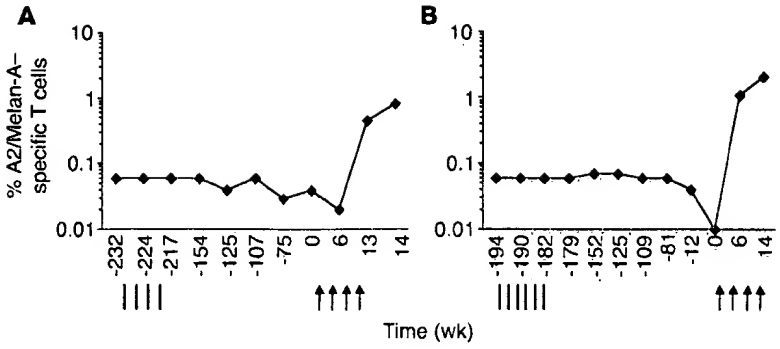

Figure 2
T cell responses to CpG vaccination (arrows) in patients that previously did not respond to vaccination with Melan-A peptide and immunological adjuvants MPL and QS21 (vertical lines). Percentages of multimer$^+$ T cells were determined ex vivo in PBMCs collected over an observation period of 4 to 5 years. (**A**) Patient LAU 321; (**B**) Patient LAU 371.

remained at stable low values (patient LAU 321, 0.05% ± 0.01%; patient LAU 371, 0.06% ± 0.02%), a frequent observation in immune nonresponders (27). Nevertheless, both patients responded rapidly and strongly to vaccination with CpG 7909, Melan-A peptide, and IFA (Figure 2).

T cell frequencies peaked 7–10 days after recall vaccination. Results in Figures 1 and 2 were obtained with blood samples collected 7–10 days following the monthly recall vaccinations (i.e., vaccinations 2 and 4). To determine the short-term kinetics of T cell responses, we analyzed additional blood samples collected immediately before and 14 days after recall vaccination with CpG 7909, Melan-A peptide, and IFA. The highest T cell levels were reached 7–10 days after recall vaccination (Figure 3). Maximal frequencies (as shown in Figures 1 and 2) were observed on day 7 in 4 patients (LAU 818, LAU 618, LAU 701, and LAU 672) and on day 10 in 3 patients (LAU 627, LAU 371, and LAU 444). Interestingly, frequencies of circulating Melan-A-specific T cells dropped to relatively low levels 14 days after recall vaccination (frequencies for patient LAU 321 were not evaluable, since blood samples from days 7 and 14 were not available). Thus, recall vaccination with CpG 7909, Melan-A peptide, and IFA led to increased T cell frequencies during the first 7–10 days, followed by a decline shortly afterwards, similarly to previous observations in mice (28–30).

Predominance of effector memory T cells expressing effector genes in vivo. It is well established that Melan-A-specific T cells in melanoma patients comprise naive and activated cells (31). To determine the differentiation state of vaccine-induced Melan-A-specific T cells, we first assessed CD45RA and CCR7 cell surface expression (32, 33) by Melan-A-specific T cells. Before vaccination, the majority were naive CCR7$^+$ CD45RA$^+$ T cells (Figure 4A). In contrast, after vaccination most Melan-A-specific T cells displayed an effector memory phenotype (CD45RA$^-$CCR7$^-$; Figure 4A and Table 1). After 4 vaccinations, effector memory cells accounted for 82% ± 13% of Melan-A multimer$^+$ T cells (mean ± SD of the 8 patients), and naive, central memory, and effector T cells were only found at low percentages. To assess effector gene expression directly, we sorted Melan-A-specific T cell subpopulations according to their expression of CD45RA and CCR7. As described previously (33), we sorted 5-cell aliquots and isolated mRNA, which was transcribed to cDNA then nonspecifically amplified and finally used for PCR with sequence-specific primers. All cells were positive for CD3 (Figure 4B). As expected, naive cells, which are not cytolytic and do not produce cytokines, did not contain detectable granzyme B, perforin, TNF-α, or NK receptor CD94 mRNA, and they only rarely gave an IFN-γ signal. After vaccination, granzyme B, perforin, and IFN-γ mRNA transcripts were found in significant fractions of 5-cell aliquots of effector memory cells. Finally, after 4 vaccinations, effector memory cells showed increased expression of granzyme B and perforin, and some fractions of 5-cell aliquots also expressed TNF-γ and NK receptor CD94.

Activated T cells with fine specificity to the natural tumor antigen. All vaccinations were done with the Melan-A analog peptide ELAGIGILTV, which contains the amino acid leucine in position 2 instead of the natural amino acid alanine. This substitution causes the analog peptide to bind more strongly and stably to HLA-A2, resulting in increased antigenicity and immunogenicity (31). To test the ability of T cells elicited by the vaccine to recognize A2/Melan-A antigen, we assessed the fine specificity of Melan-A-specific T cells in IFN-γ Elispot assays performed ex vivo. After vaccination, Melan-A-specific, IFN-γ-producing T cells from all 8 patients reached frequencies above the detection limit of 0.01% of CD8$^+$ T cells (Figure 5A and Table 2). The natural and analog peptides triggered comparable frequencies of Elispot-forming cells (0.12% ± 0.12% and 0.14% ± 0.10%, respectively). To assess the functional avidity, we tested the cytotoxic activity of A2/Melan-A-specific T cell clones derived from patients after vaccination. Cytotoxicity was peptide-specific and occurred at low peptide concentrations (Figure 5B), demonstrating high functional avidity to A2/Melan-A. The observed hierarchy of peptide recognition efficiency is characteristic for the majority of Melan-A-specific T cell clones and is in accordance with the hierarchy of binding strength of the 3 peptide variants to HLA-A*0201 (31). In agreement with these findings, the clones indeed killed the A2$^+$/Melan-A$^+$ melanoma cell line Me 275, but not the A2$^+$/Melan-A$^-$ melanoma cell line NA8 (Figure 5C). These data

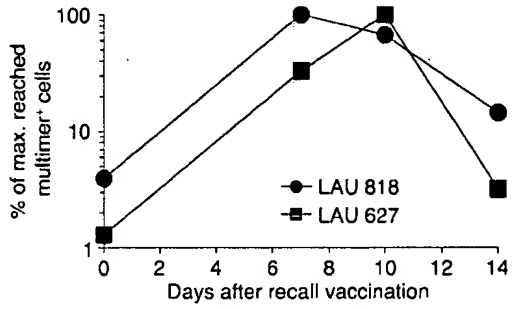

Figure 3
Short-term kinetics of Melan-A–specific T cells from 2 representative patients with maximal (max.) responses on day 7 (patient LAU 818) and on day 10 (patient LAU 627) after recall vaccination with CpG 7909, Melan-A peptide, and IFA. PBMCs were collected immediately before, as well as 7, 10, and 14 days after, recall vaccination. Percentages of multimer$^+$ T cells were determined ex vivo and calculated in percentages of maximally reached values (100%) per patient.

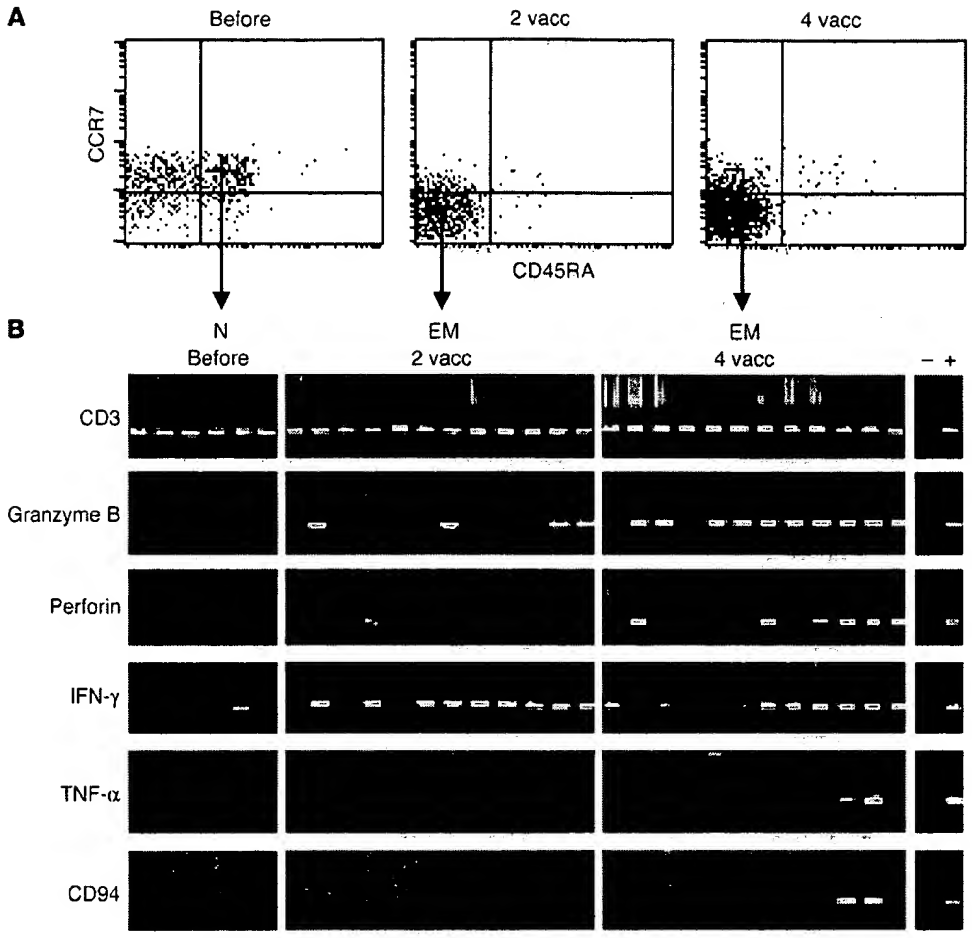

Figure 4
Expression of effector mediators by vaccine-activated Melan-A–specific T cells. Multiparameter cytometric analysis and sorting was performed with A2/Melan-A multimers and antibodies specific for CD8, CD45RA, and CCR7. (**A**) Expression of CD45RA and CCR7 is shown for A2/Melan-A multimer$^+$ CD8$^+$ gated cells. (**B**) RT-PCR gene expression analysis was performed (33) using primers specific for CD3, granzyme B, perforin, IFN-γ, TNF-α, and CD94, on sorted A2/Melan-A multimer$^+$ T cells which were RA$^+$CCR7$^+$ (naive cells) or RA$^-$CCR7$^+$ effector memory cells. Each band represents the RT-PCR product from RNA isolated from sorted 5-cell aliquots. Data in **A** and **B** (representative of 8 and 2 patients analyzed, respectively) are from PBMCs collected from patient LAU 371 before and after 2 and 4 vaccinations. +, positive control; –, negative control; N, naive T cells; EM, effector memory T cells.

demonstrate that T cells induced by a vaccine comprised of a synthetic Melan-A analog peptide specifically recognized the natural Melan-A antigen expressed by tumor cells.

Discussion

This study represents the first human trial of CpG ODN combined with a T cell peptide antigen. It demonstrates that CpG 7909 is an efficient adjuvant that promotes rapid antigen-specific CD8$^+$ T cell responses. As compared to vaccination without CpG, responses were 1 order of magnitude higher. Synthetic peptide plus adjuvant vaccines have been shown to induce only low frequencies of circulating antigen-specific T cells, despite administration of 8 or more injections (34, 35). Moreover, in the majority of cancer patient studies reported so far, T cell responses could not be detected ex vivo but rather only after 1 or more rounds of in vitro T cell stimulation and proliferation (21, 24, 36, 37). A small number of studies (using 0.5–1 mg peptide per vaccination plus adjuvants or cytokines) described ex vivo detectable T cell responses (22, 25), but T cell frequencies were still 10–100 times lower than what we observed in the present study. Only 1 study reported higher percentages of antigen-specific T cells following vaccinations with the particularly immunogenic gp100 T2M analog peptide and IFA. However, very high doses were used for vaccination: the cumulative peptide dose was 100-fold higher and the number of vaccinations 10-fold higher than in our study (38).

The HLA-A2/Melan-A antigenic system constitutes a well-defined model for studies of spontaneous and vaccine-induced CD8$^+$ T cell responses in humans (31, 37). Besides the gp100 analog peptide 2TM, Melan-A peptide is one of the rare cancer peptide antigens capable of inducing ex vivo detectable T cell responses in relatively large proportions of patients (22, 37). The availability of strong adjuvants such as CpG 7909 makes it possible to investigate the immunogenicity of weaker antigens, which make up the majority of known cancer epitopes.

Both CpG and peptide were given at low doses. Future studies will test whether increased doses of CpG 7909 and/or peptides can further enhance T cell activation. CpG 7909 can be administered to humans with generally acceptable tolerability at doses up to at least 20 mg per injection weekly for 6 months or longer (A. Krieg, unpublished observations), that is, at cumulative doses that are 250-fold higher than in our study. Very high CpG ODN doses (2.5 mg/kg) given daily for 20 days have recently been reported to destroy lymphoid tissue structures in mice (39). High-dose effects also include extramedullary hematopoeisis and a lethal systemic inflammatory response syndrome in mice (40). The potential toxicities of high-dose CpG 7909 administration in humans are likely to be largely different from those reported in mice, due to the much more restricted distribution of TLR9 expression in human as compared to mouse immune cells (5, 17). Our patients had no apparent liver toxicity, no enlargement of lymph nodes or spleen, and intact antigen-specific B

Table 1
Percentages of naive and non-naive Melan-A–specific T cells

	T_N	T_{CM}	T_{EM}	T_E
CD45RA	+	–	–	+
CCR7	+	+	–	–
LAU 627	0	4	93	2
LAU 818	0.7	7	89	3
LAU 618	0.1	8	91	0.8
LAU 321	5	30	60	5
LAU 371	0.7	15	84	0.6
LAU 444	1	1	93	5
LAU 701	2	5	62	31
LAU 672	5	7	86	3
Mean	2	10	82	6
SD	2	9	13	9

Percentages of naive (T_N; RA^+CCR7^+), central memory (T_{CM}; RA^-CCR7^+), effector memory (T_{EM}; RA^-CCR7^-), and effector (T_E; RA^+CCR7^-) T cells were determined among A2/Melan-A multimer$^+$ gated T cells from PBMCs collected 7–10 days after the fourth vaccination.

cell responses (data not shown). Two lymph node biopsies after CpG 7909 vaccination showed follicles with normal structure. Interestingly, 4 of 8 patients developed circulating anti-dsDNA antibodies as detected by ELISA, but there were no clinical signs for autoimmune disease, and systemic inflammatory markers in sera (IP-10, CRP) remained normal. We are currently investigating the possibility that the antibodies could be specific for CpG 7909.

Out of the 8 patients included in the study, 3 had no evidence for disease at study entry; during the study, 1 of them remained disease-free, 1 progressed, and 1 showed local tumor relapse. Among the 5 patients with measurable disease at study entry, 1 had stable disease, 2 progressed, and 2 showed local relapse. This phase I study was not designed to assess tumor response, and the follow-up time was short (mean of 7 ± 2 months after inclusion in the study). Comprehensive clinical results need to be obtained in phase II trials with more patients and a longer follow-up time.

In contrast to mouse TLR9, human TLR9 is only expressed by pDCs and B cells. Presumably, B cells are not involved in T cell activation, suggesting that pDCs were responsible for the observed T cell responses (41). One important question is whether our vaccine was capable of priming T cells, or whether the observed responses were due to reactivation and/or redistribution of previously primed cells. Remarkably, the levels of circulating naive Melan-A-specific T cells were abnormally low after CpG 7909 vaccination (undetectable in 4 patients, 0.03% ± 0.02% in the remaining 4 patients). For comparison, naive Melan-A-specific T cells are present at relatively high frequencies (0.07% ± 0.06%) in untreated healthy individuals and melanoma patients (42). Our observations that all patients responded, and that naive cell frequencies were low after vaccination, suggest that CpG 7909 vaccination was indeed capable of priming naive T cells. This conclusion is also supported by recent mouse experiments showing that CpG ODN–matured pDCs can prime $CD8^+$ T cells in vivo (41).

We also analyzed whether the prevaccination immune status differed between patients treated with and without CpG. However, we did not find significant differences with regard to prevaccination frequencies of naive cells or of each of the 3 other Melan-A-specific T cell populations defined by CD45RA/CCR7 expression. Data were interpretable from 3 patients of each group (data not shown). We have recently demonstrated that the percentage of preexisting CD28 negative Melan-A-specific T cells correlates with T cell responsiveness to vaccination with peptide and IFA in stage III–IV melanoma patients (26). Analyzing this aspect in the present study, we could not find a statistically significant difference between the 2 patient groups (mean prevaccination percentages ± SD of CD28 negative Melan-A-specific T cells were 28% ± 33% and 29% ± 27% in patients treated with and without CpG, respectively). Data were interpretable from 6 patients of each group (data not shown). Based on these findings, one may speculate that vaccination with CpG induces increased frequencies of circulating T cells independently of the patient's prevaccination immune status, whereas T cell responses to peptide and IFA vaccination (without CpG) depend significantly on endogenous (tumor-driven) immunogenicity (26).

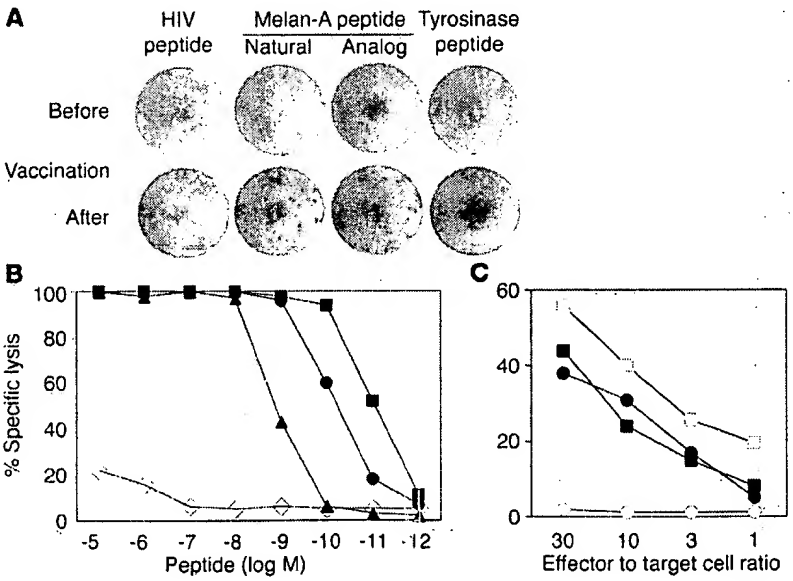

Figure 5
T cell receptor fine-specificity and tumor cell recognition. (**A**) PBMCs from patient LAU 371 were tested ex vivo in IFN-γ Elispot assays before and after vaccination with HIV, tyrosinase, and Melan-A natural and analog peptides. (**B**) Melan-A–specific T cells were sorted by flow cytometry and cloned, and cytotoxicity was tested against T2 cells in the presence of the following titrated HLA-A2 binding peptides: Melan-A analog (squares), Melan-A natural decamer (circles), Melan-A natural nonamer (triangles), and influenza matrix protein GILGFVFTL (diamonds). (**C**) Cytotoxicity against $HLA\text{-}A2^+$ melanoma cell lines Me 275 (Melan-A^+; filled symbols) and NA8 (Melan-A^-; open symbols) in the presence (squares) or absence (circles) of synthetic Melan-A analog peptide. Data shown were generated with clone 6 derived from patient LAU 371 and are representative for 12 of 22 clones generated from patients LAU 371 and LAU 444.

Table 2
Percentage of IFN-γ Elispot–forming cells

	Melan-A peptide	
Patient	**Natural**	**Analog**
LAU 627	0.08	0.08
LAU 818	0.05	0.06
LAU 618	0.07	0.13
LAU 321	0.02	≤0.01
LAU 371	0.11	0.12
LAU 444	0.40	0.32
LAU 701	0.02	0.02
LAU 672	0.19	0.23
Mean	0.12	0.14
SD	0.12	0.10

Percentages of Elispot-forming cells among $CD8^+$ T cells in PBMCs are shown for all 8 patients after vaccination. Background results with HIV polymerase peptide and without peptide were below the detection limit of 0.01% (not shown).

The pattern of specific T cell expansion and contraction following recall vaccination is reminiscent of acute T cell responses observed in mice (28–30). Interestingly, in patients vaccinated with Melan-A and IFA without CpG, such rapid changes of specific T cell frequencies were not observed (data not shown). In the majority of cancer vaccine studies reported so far, antigen-specific T cells were analyzed at only a single time point after each vaccination. With the introduction of highly efficient adjuvants such as CpG 7909, stronger, shorter-lasting T cell responses can be observed. Thus, antigen-specific T cells should be monitored at multiple time points after vaccination.

One possible explanation for the rapid decline during the second week after recall vaccination is T cell migration into lymphoid and tumor tissues (43). We were able to investigate metastases surgically removed from 3 patients after vaccination with CpG 7909, Melan-A, and IFA, and found that 5.03%, 0.12%, and 0.08% of $CD8^+$ T cells were specific for Melan-A (data not shown). Ongoing studies will address the question of whether vaccine-activated T cell clones are found in these lesions. We are also developing techniques to estimate the percentage of T cells that undergo apoptosis and/or migrate to other compartments (29, 44).

The vaccine-induced T cell populations consisted predominantly of effector memory T cells ($CD45RA^-CCR7^-$). Central memory and effector cells were also induced, but to a much lesser extent. Most non-naive cells expressed IFN-γ mRNA. Effector memory cells expressed granzyme B mRNA, and some of them expressed perforin mRNA. While this was the case for the majority of 5-cell aliquots after 4 vaccinations, only a few cells were positive for granzyme B after only 2 vaccinations. Finally, TNF-α and CD94 were expressed by only a small fraction of cells. Through our ongoing studies, we aim to elucidate whether multiple recall vaccinations can induce effector functions in the majority of cells.

It is conceivable that the magnitude and durability of antigen-specific T cell responses induced by peptide-based vaccines could be further enhanced by the use of other types of CpG ODNs, such as the C type, which recently has been reported to induce higher levels of IFN-α secretion than the B type, which was used in this study (45–48). Indeed, IFN-α appears to improve the development of T cell memory (49, 50). Moreover, various other TLR ligands are becoming available for use in humans and are interesting candidates as adjuvants for vaccination and induction of protective immunity.

In conclusion, this study underscores the synergy of combined triggering of innate and specific immunity. The TLR9 ligand CpG 7909 is a potent stimulator of innate immune cells, and its coadministration with antigen induces high numbers of antigen-specific $CD8^+$ T cells. Our findings suggest that further approaches to T cell–directed immunotherapy should take advantage of such a synergy.

Methods

Patients, eligibility criteria, and study protocol. $HLA\text{-}A2^+$ patients with histologically proven metastatic melanoma of the skin expressing Melan-A/MART-1 (determined by RT-PCR or immunohistochemistry) were included after informed consent was obtained in this phase I prospective trial of the Ludwig Institute for Cancer Research (LICR). Study protocols were approved by the LICR protocol review committee, as well as by the medical and ethical committees of the University Hospital (Lausanne, Switzerland). Inclusion criteria were as follows: Karnofsky performance status of at least 70%, normal complete blood count and kidney-liver function, and no concomitant anti-tumor therapy or immunosuppressive drugs. Exclusion criteria were pregnancy, seropositivity for HIV-1 Ab or hepatitis B surface antigen, brain metastasis, uncontrolled bleeding, clinically significant autoimmune disease, or New York Heart Association class III–IV heart disease. Study end points were toxicity and $CD8^+$ T cell response. All patients were evaluated for immune response and treatment toxicity. Patients received 4 vaccinations subcutaneously in monthly intervals. The low-dose vaccines were composed of 500 μg CpG 7909, 100 μg Melan-A analog peptide, and 300 μl IFA (Montanide ISA-51; Seppic) all mixed together and prepared in a syringe as a stable emulsion. Phosphorothioate backbone CpG 7909 (TCGTCGTTTTGTCGTTTTGTCGTT) was produced by Coley Pharmaceutical Group under good manufacturing practice conditions, and had no detectable endotoxin by limulus amebocyte lysate assay. The Melan-A analog $peptide_{26\text{-}35}$ ELAGIGILTV was synthesized by NeoMPS Inc. and formulated (330 μg/ml in PBS/30% DMSO) by the Biological Production Facility, LICR Melbourne. The IFA (Montanide ISA-51; Seppic) contained mineral oil (Drakeol) and anhydro mannitol octadecanoate. Control patients were treated similarly with 4 monthly vaccinations as described previously (23), except the vaccines did not contain CpG ODN. As in most phase I studies, patients were not randomized, but first assigned to the control group and subsequently to the CpG group. Patient LAU 444 was first included in the control group, and 2 years later in the CpG group.

Blood cells, HLA-A2/peptide multimers, flow cytometry, and IFN-γ Elispot assays. Ficoll-Paque centrifuged PBMCs (1×10^7–2×10^7) were cryopreserved in RPMI 1640, 40% FCS, and 10% DMSO. Phycoerythrin-labeled HLA-A*0201/peptide multimers (originally called tetramers) were prepared as described previously (42, 51) with Melan-A analog $peptide_{26\text{-}35}$ ELAGIGILTV. Anti-CD8, anti-CD28, and allophycocyanin-conjugated goat anti-rat IgG were purchased from BD Biosciences and anti-CD45RA was from Immunotech. Anti-CCR7 rat IgG2a mAb 3D12 was provided by Martin Lipp (Max Delbrueck Center for Molecular Medicine, Berlin, Germany). Five color stains were done with HLA-A2/peptide multimers, FITC-conjugated anti-CD28, PE–Texas Red–conjugated anti-CD45RA, allophycocyanin-Cy7–conjugated anti-CD8 reagents, and anti-

CCR7 mAb followed by allophycocyanin-conjugated goat anti-rat IgG antibody. Briefly, $CD8^+$ T cells were enriched using a MiniMACS device (Miltenyi Biotec) resulting in more than 90% $CD3^+CD8^+$ cells. Cells (10^6) were incubated with multimers (1 μg/ml, 60 minutes, room temperature) and then with antibodies (30 minutes, 4°C). We acquired 5×10^5 $CD8^+$ T cells per sample with a FACSVantage machine, and data were analyzed with CellQuest software (BD Biosciences). IFN-γ Elispot assays were performed using IFN-γ–specific antibodies (Diaclone, Biotest). Briefly, plates were coated overnight with antibody to human IFN-γ and washed 6 times. We added 1.66×10^5 PBMCs/well in 200 μl Iscove medium (Gibco; Invitrogen Corp.) supplemented with 8% human serum and 10 μg/ml peptide and incubated it for 16 hours at 37°C. Assays were performed in 6 replicates, without peptide or with peptides derived from Melan-A (natural EAAGIGILTV, analog ELAGIGILTV), tyrosinase YMDGTMSQV, and HIV-1 polymerase ILKEPVHGV. Cells were removed, and plates were developed with a second (biotinylated) antibody to human IFN-γ and streptavidin-alkaline phosphatase (Diaclone, Biotest). The spots were revealed with BCIP/NBT substrate (Sigma Tablets; Sigma-Aldrich) and counted with an automatic reader (Bioreader 2000; BioSys GmbH). The percentage of $CD3^+CD8^+$ PBMCs was determined by flow cytometry on the same batch of cryopreserved cells. Results of both multimer$^+$ T cells and Elispot-forming T cells were calculated as a percentage of $CD8^+$ T cells. For each patient and assay system, pre- and post-vaccination samples were thawed and tested in the same experiment.

Quality control of laboratory immune monitoring. Standardization of multimer and IFN-γ Elispot assays was done with 180 unselected blood samples from healthy donors and patients (27). For both multimer and IFN-γ Elispot assays, the detection limit was 100 cells in 10^6 $CD8^+$ T cells (0.01%) (42). Repeated analyses showed 15% ± 16% (mean ± SD) variation in multimer$^+$ cells (37). Good reproducibility was also found for the Elispot assay (variation of 30% ± 21%). In addition, longitudinal intraindividual result variability was assessed by testing multiple blood samples from individuals without a T cell response against Melan-A. Variation coefficients (SD in percent of mean multimer$^+$ values) were 20% ± 27%. For IFN-γ Elispot assays, negative controls with all PBMC samples had a mean of less than 0.003% spots among $CD8^+$ T cells, confirming that the background was far below the detection limit of 0.01%.

Cell sorting, cDNA amplification, and 5-cell RT-PCR. $CD8^+$ T cells were enriched with a MiniMACS device and stained with multimers and antibodies as described above. Five-cell aliquots were sorted directly with a FACSVantage SE machine into wells of 96-V bottom plates. cDNA preparation, cDNA amplification, and PCR were done using primers for CD3, granzyme B, perforin, IFN-γ, TNF-γ, and CD94 as described (33). Either water or Daudi B cell line extract was used as a negative PCR control (–); 10^3 PBMCs from a healthy individual were used as a positive control (+).

T cell cloning and cytotoxicity assay. Multimer$^+$ $CD8^+$ T cells were sorted by flow cytometry, cloned by limiting dilution, and expanded with phytohemagglutinin (PHA) and allogenic feeder cells in medium containing 150 U/ml human recombinant IL-2 (hrIL-2) and 10 ng/ml hrIL-7. Subsequently, they were periodically (every 3–4 weeks) restimulated with PHA, irradiated feeder cells, and hrIL-2. Lytic activity and antigen recognition were assessed functionally in 4-hour ^{51}Cr release assays (42). Target cells were T2 cells ($A2^+$/Melan-A^-) and the melanoma cell lines Me 275 ($A2^+$/Melan-A^+) and NA8 ($A2^+$/Melan-A^-) (31). The percentage of specific lysis was calculated as follows: 100 × ([experimental – spontaneous release]/[total – spontaneous release]).

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1. Janeway, C.A., Jr. 1989. Approaching the asymptote? Evolution and revolution in immunology. *Cold Spring Harb. Symp. Quant. Biol.* **54**:1–13.
2. Marrack, P., and Kappler, J. 1994. Subversion of the immune system by pathogens. *Cell.* **76**:323–332.
3. Medzhitov, R., and Janeway, C.A., Jr. 1997. Innate immunity: impact on the adaptive immune response. *Curr. Opin. Immunol.* **9**:4–9.
4. Akira, S., Takeda, K., and Kaisho, T. 2001. Toll-like receptors: critical proteins linking innate and acquired immunity. *Nat. Immunol.* **2**:675–680.
5. Kadowaki, N., et al. 2001. Subsets of human dendritic cell precursors express different toll-like receptors and respond to different microbial antigens. *J. Exp. Med.* **194**:863–869.
6. Steinman, R.M. 1991. The dendritic cell system and its role in immunogenicity. *Annu. Rev. Immunol.* **9**:271–296.
7. Klinman, D.M. 2004. Immunotherapeutic uses of CpG oligodeoxynucleotides. *Nat. Rev. Immunol.* **4**:249–259.
8. Hemmi, H., et al. 2000. A Toll-like receptor recognizes bacterial DNA. *Nature.* **408**:740–745.
9. Takeshita, F., et al. 2001. Cutting edge: Role of Toll-like receptor 9 in CpG DNA-induced activation of human cells. *J. Immunol.* **167**:3555–3558.
10. Weiner, G.J., Liu, H.M., Wooldridge, J.E., Dahle, C.E., and Krieg, A.M. 1997. Immunostimulatory oligodeoxynucleotides containing the CpG motif are effective as immune adjuvants in tumor antigen immunization. *Proc. Natl. Acad. Sci. U. S. A.* **94**:10833–10837.
11. Lipford, G.B., et al. 1997. CpG-containing synthetic oligonucleotides promote B and cytotoxic T cell responses to protein antigen: a new class of vaccine adjuvants. *Eur. J. Immunol.* **27**:2340–2344.
12. Oxenius, A., Martinic, M.M., Hengartner, H., and Klenerman, P. 1999. CpG-containing oligonucleotides are efficient adjuvants for induction of protective antiviral immune responses with T-cell peptide vaccines. *J. Virol.* **73**:4120–4126.
13. Vabulas, R.M., Pircher, H., Lipford, G.B., Hacker, H., and Wagner, H. 2000. CpG-DNA activates in vivo T cell epitope presenting dendritic cells to trigger protective antiviral cytotoxic T cell responses. *J. Immunol.* **164**:2372–2378.
14. Davila, E., and Celis, E. 2000. Repeated administration of cytosine-phosphorothiolated guanine-containing oligonucleotides together with peptide/protein immunization results in enhanced CTL responses with anti-tumor activity. *J. Immunol.*

165:539–547.
15. Miconnet, I., et al. 2002. CpG are efficient adjuvants for specific CTL induction against tumor antigen-derived peptide. *J. Immunol.* **168**:1212–1218.
16. Hartmann, G., et al. 2000. Delineation of a CpG phosphorothioate oligodeoxynucleotide for activating primate immune responses in vitro and in vivo. *J. Immunol.* **164**:1617–1624.
17. Bauer, S., et al. 2001. Human TLR9 confers responsiveness to bacterial DNA via species-specific CpG motif recognition. *Proc. Natl. Acad. Sci. U. S. A.* **98**:9237–9242.
18. Krieg, A.M. 2002. CpG motifs in bacterial DNA and their immune effects. *Annu. Rev. Immunol.* **20**:709–760.
19. Davis, H.L., et al. 2000. CpG DNA overcomes hyporesponsiveness to hepatitis B vaccine in orangutans. *Vaccine.* **18**:1920–1924.
20. Halperin, S.A., et al. 2003. A phase I study of the safety and immunogenicity of recombinant hepatitis B surface antigen co-administered with an immunostimulatory phosphorothioate oligonucleotide adjuvant. *Vaccine.* **21**:2461–2467.
21. Cormier, J.N., et al. 1997. Enhancement of cellular immunity in melanoma patients immunized with a peptide from MART-1/Melan A. *Cancer J. Sci. Am.* **3**:37–44.
22. Pullarkat, V., et al. 2003. A phase I trial of SD-9427 (progenipoietin) with a multipeptide vaccine for resected metastatic melanoma. *Clin. Cancer Res.* **9**:1301–1312.
23. Liénard, D., et al. 2004. Ex vivo detectable activation of Melan-A specific T cells correlating with inflammatory skin reactions in melanoma patients vaccinated with peptides in IFA. *Cancer Immunity.* **4**:4.
24. Rosenberg, S.A., et al. 1998. Immunologic and therapeutic evaluation of a synthetic peptide vaccine for the treatment of patients with metastatic melanoma. *Nat. Med.* **4**:321–327.
25. Schaed, S.G., et al. 2002. T-cell responses against tyrosinase 368-376(370D) peptide in HLA*A0201+ melanoma patients: randomized trial comparing incomplete Freund's adjuvant, granulocyte macrophage colony-stimulating factor, and QS-21 as immunological adjuvants. *Clin. Cancer Res.* **8**:967–972.
26. Speiser, D.E., et al. 2003. Disease-driven T cell activation predicts immune responses to vaccination against melanoma. *Cancer Immunity.* **3**:12.
27. Speiser, D.E., et al. 2004. Ex vivo analysis of human antigen specific CD8+ T cell responses: Quality assessment of fluorescent HLA-A2 multimers and IFN gamma Elispot assays for patient immune monitoring. *J. Immunother.* **27**:298–308.
28. Kawabe, Y., and Ochi, A. 1991. Programmed cell death and extrathymic reduction of Vβ8+ CD4+ T cells in mice tolerant to Staphylococcus aureus enterotoxin B. *Nature.* **349**:245–248.
29. Lenardo, M., et al. 1999. Mature T lymphocyte apoptosis-immune regulation in a dynamic and unpredictable antigenic environment. *Annu. Rev. Immunol.* **17**:221–253.
30. Blattman, J.N., et al. 2003. Therapeutic use of IL-2 to enhance antiviral T-cell responses in vivo. *Nat. Med.* **9**:540–547.
31. Romero, P., et al. 2002. Antigenicity and immunogenicity of Melan-A/MART-1 derived peptides as targets for tumor reactive CTL in human melanoma. *Immunol. Rev.* **188**:81–96.
32. Sallusto, F., Lenig, D., Forster, R., Lipp, M., and Lanzavecchia, A. 1999. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. *Nature.* **401**:708–712.
33. Rufer, N., et al. 2003. Ex vivo characterization of human CD8+ T subsets with distinct replicative history and partial effector functions. *Blood.* **102**:1779–1787.
34. Boon, T., and Van den Eynde, B. 2003. Tumour immunology. *Curr. Opin. Immunol.* **15**:129–130.
35. Monsurro, V., et al. 2001. Kinetics of TCR use in response to repeated epitope-specific immunization. *J. Immunol.* **166**:5817–5825.
36. Jaeger, E., et al. 1996. Generation of cytotoxic T cell responses with synthetic melanoma associated peptides in vivo, implications for tumor vaccines with melanoma associated antigens. *Int. J. Cancer.* **66**:162.
37. Speiser, D.E., et al. 2003. Evaluation of melanoma vaccines with molecularly defined antigens by ex vivo monitoring of tumor specific T cells. *Semin. Cancer Biol.* **13**:461–472.
38. Powell, D.J., Jr., and Rosenberg, S.A. 2004. Phenotypic and functional maturation of tumor antigen-reactive CD8+ T lymphocytes in patients undergoing multiple course peptide vaccination. *J. Immunother.* **27**:36–47.
39. Heikenwalder, M., et al. 2004. Lymphoid follicle destruction and immunosuppression after repeated CpG oligodeoxynucleotide administration. *Nat. Med.* **10**:187–192.
40. Sparwasser, T., et al. 1999. Immunostimulatory CpG-oligodeoxynucleotides cause extramedullary murine hemopoiesis. *J. Immunol.* **162**:2368–2374.
41. Salio, M., Palmowski, M.J., Atzberger, A., Hermans, I.F., and Cerundolo, V. 2004. CpG-matured murine plasmacytoid dendritic cells are capable of in vivo priming of functional CD8 T cell responses to endogenous but not exogenous antigens. *J. Exp. Med.* **199**:567–579.
42. Pittet, M.J., et al. 1999. High frequencies of naive Melan-A/MART-1-specific CD8+ T cells in a large proportion of human histocompatibility leukocyte antigen (HLA)-A2 individuals. *J. Exp. Med.* **190**:705–715.
43. Yu, P., et al. 2004. Priming of naive T cells inside tumors leads to eradication of established tumors. *Nat. Immunol.* **5**:141–149.
44. Houghton, A.N. 2004. LIGHTing the way for tumor immunity. *Nat. Immunol.* **5**:123–124.
45. Verthelyi, D., Ishii, K.J., Gursel, M., Takeshita, F., and Klinman, D.M. 2001. Human peripheral blood cells differentially recognize and respond to two distinct CPG motifs. *J. Immunol.* **166**:2372–2377.
46. Hartmann, G., et al. 2003. Rational design of new CpG oligonucleotides that combine B cell activation with high IFN-alpha induction in plasmacytoid dendritic cells. *Eur. J. Immunol.* **33**:1633–1641.
47. Marshall, J.D., et al. 2003. Identification of a novel CpG DNA class and motif that optimally stimulate B cell and plasmacytoid dendritic cell functions. *J. Leukoc. Biol.* **73**:781–792.
48. Vollmer, J., et al. 2004. Characterization of three CpG oligodeoxynucleotide classes with distinct immunostimulatory activities. *Eur. J. Immunol.* **34**:251–262.
49. Akbar, A.N., Lord, J.M., and Salmon, M. 2000. IFN-alpha and IFN-beta: a link between immune memory and chronic inflammation. *Immunol. Today.* **21**:337–342.
50. Sprent, J., and Surh, C.D. 2002. T cell memory. *Annu. Rev. Immunol.* **20**:551–579.
51. Altman, J.D., et al. 1996. Phenotypic analysis of antigen-specific T lymphocytes. *Science.* **274**:94–96.

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Co-administration of CpG oligonucleotides enhances the late affinity maturation process of human anti-hepatitis B vaccine response

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Abstract

We assessed the avidity maturation process elicited by human immunization with alum-adsorbed HBsAg alone or with a novel adjuvant containing CpG motifs (CpG 7909). Mean avidity indexes and distribution of low- and high-avidity anti-HBs indicated that avidity maturation essentially takes place late after priming. CpG 7909 markedly enhanced this affinity maturation process, increasing the pool of high-avidity antibodies. The influence of CpG 7909 was antigen-specific, isotype-specific and distinct from the influence on anti-HBs production, as avidity did not correlate with anti-HBs IgG titers. This is the first demonstration that a novel human adjuvant may induce antibodies with higher antigen-binding affinity.

Keywords: Human; B lymphocytes; Antibodies

1. Introduction

Affinity maturation of serum antibody is an essential and yet not fully understood feature of humoral responses to T-dependent antigens. Complex cellular events lead to the preferential generation of high affinity antibody secreting cells (ASC) and their accumulation as long lived ASC in the bone marrow (BM). It is well known that, early after antigen exposure, B cells are induced to proliferate and develop into ASC within extrafollicular foci. These ASC produce antibodies encoded by germline immunoglobulin genes and are most often of relatively low affinity [1,2]. Subsequently, some proliferating B cells enter B cell follicles and give rise to germinal centers (GC), in which somatic hypermutation of V(D)J immunoglobulin genes [3,4] and competition for antigen retained on GC follicular dendritic cells (FDC) [5] concur, leading to the selection of B cells of the highest affinity [6–8]. This affinity maturation process results in increased numbers of ASC that produce antibodies with progressively higher affinities and faster on-rates [9–12]. Accordingly, total antibody affinity to large complex antigens (i.e. functional

Abbreviations: ASC, antibody secreting cells; anti-HBs, HBsAg-specific antibody; AI, avidity index; BM, bone-marrow; CpG oligos, oligonucleotides containing CpG motifs; FDC, follicular dendritic cells; GC, germinal centers; TT, tetanus toxoid

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affinity, or avidity [13]) progressively increases. It should be specified that whereas affinity refers to the strength of binding between a single antigenic determinant and an individual antibody combining site (sum of the attractive and repulsive forces), avidity refers to the overall strength of binding between an antigen with many antigenic determinants and multivalent antibodies. The avidity is influenced by both the valence of the antibody and the valence of the antigen, is more than the sum of the individual affinities, and is most directly relevant to function. This avidity maturation process, which may be essential for protective efficacy, has not been characterized in detail following human immunization with T-dependent protein antigens, and as such, important questions remain regarding its efficacy, kinetics and importance in relation to vaccination strategies.

Immunostimulatory DNA sequences containing unmethylated CpG dinucleotides in the context of particular base sequences (CpG motifs) exert a strong stimulatory influence on the immune system. Such sequences, which are either found naturally in bacterial DNA or produced as synthetic oligodeoxynucleotides (CpG oligos), directly activate human B cells and plasmacytoid dendritic cells (pDC) via toll-like receptor 9 (TLR-9). CpG oligos act as polyclonal activators which directly activate B cells to proliferate and differentiate into IgG producing cells, and somewhat prevent mature B cells from undergoing spontaneous apoptosis in vitro. CpG oligos also indirectly activate other cells such as monocytes and macrophages to produce a variety of pro-inflammatory cytokines, and in particular those associated with Th1 responses (reviewed in [14–20]). In accordance with these stimulatory influences, CpG oligos were identified as potent vaccine adjuvants, capable of enhancing $CD4^+$, $CD8^+$ cytotoxic and Ab responses to a wide variety of antigens both in adult (reviewed in [21,22]) and neonatal [23–25] animal immunization models.

As a result of their strong adjuvanticity and low reactogenicity, CpG oligos are currently considered as one of the most promising adjuvants for the development of future vaccines against diverse conditions including infectious diseases, allergies or cancer [26,27]. CpG 7909, a 24-mer oligo containing three CpG motifs, has been identified for its potent ability to stimulate human immune cells and recently entered first human testing as a saline solution. In this double-blind randomized phase I study, CpG 7909 was tested for safety as well as its ability to augment responses of healthy volunteers to a prophylactic vaccine against hepatitis B (HBV). Subjects in the experimental groups, who received the HBV vaccine with CpG 7909 added, demonstrated significantly higher levels of antibodies against HBsAg (anti-HBs) than subjects in the control group (manuscript in preparation). To identify the stage(s) of Ag-specific B cell differentiation at which CpG 7909 exerts its influence, we compared in this study the kinetics and strength of the avidity maturation process of anti-HBs antibodies induced either with an alum-adjuvanted HBV vaccine or with the same vaccine mixed with CpG.

2. Material and methods

2.1. Subjects, vaccines and immunization procedures

This phase I study was conducted at The Toronto Western Hospital and The Ottawa Hospital, Canada, as approved by the Institution Ethical Review Boards. Eligible subjects were healthy male and female adult volunteers, age 18–35, who were HBV vaccine naïve, as well as negative for hepatitis B surface antigen (HBsAg) or HBsAg-specific antibody (anti-HBs). Fifty-six subjects were enrolled and scheduled for immunization at 0, 4 and 24 weeks. The vaccine administered was Engerix-B® (GlaxoSmithKline, Rixensart, BE), containing 20 μg of alum-adsorbed HBsAg. Engerix-B® was administered alone ($n = 14$) or with admixed CpG 7909 (a 24-mer oligo containing three CpG motifs) given at doses of 0.5 mg ($n = 18$), 1.0 mg ($n = 12$) or 0.125 mg ($n = 12$). Subjects were enrolled into three cohorts which were initiated sequentially according to dose level of CpG 7909 and were then randomized to receive vaccine alone (control) or with the addition of CpG 7909 (experimental). Subjects were followed for safety and tolerability of the compound. The most frequently reported adverse events were injection site reactions, flu-like symptoms and headache. While these were significantly more frequent in CpG 7909 groups than in the control group (manuscript submitted), most were reported to be of mild to moderate intensity regardless of group. Subjects provided blood samples for assessment of HBV-specific immune response at regular intervals before and after each vaccine dose. Frozen plasma aliquots harvested 0, 8, 24, 26 and 52 weeks after priming were sent to the Center for Vaccinology (University of Geneva) for blinded determination of HBsAg-specific avidity and of tetanus (TT) specific antibody titers.

2.2. Quantification of anti-HBs and anti-tetanus Ab

HBsAg-specific IgG1 and IgG3 Ab were determined by ELISA. Plates were coated with 1 μg/ml of HBsAg, ad subtype (International Enzymes Inc., CA), overnight at room temperature. After washing and blocking, serial dilutions of sera were added to the plates and incubated at 37 °C for 60 min. The plates were then washed and incubated with peroxidase-conjugated goat anti-human IgG1 or biotin-conjugated goat anti-human IgG3 antibodies (Zimed Laboratories, San Francisco, CA) for 60 min at 37 °C, washed again and incubated directly with ABTS substrate (for IgG1) or with extravidin-peroxidase (for IgG3) followed by substrate. Antibody concentrations were calculated with the Softmax^R PRO software (Molecular Devices) by comparison with standard curves (4-parameter fitting) using in-house standards tested by end-point dilution. It should be noted that as a result of a high sensitivity of the end-point dilution IgG1/3 ELISA assay, IgG1- or IgG3-specific anti-HBs titers correspond to significantly lower values of EIA-measured anti-HBsAg IgG Ab. Antibody

concentrations to TT were determined by ELISA on plates coated with TT (Aventis Pasteur). Incubation of plasma samples was followed by successive addition of biotinylated goat anti human IgG (Sigma) and extravidin-peroxidase and ABTS substrate. Antibody concentrations were calculated using international standards of reference. Antibodies below the cut-off of the assay (100 EIU/ml for anti-HBsAg IgG1, 50 EIU/ml for anti-HBsAg IgG3, 100 mIU/ml for anti-tetanus IgG) were given an arbitrary titer of one half of the cut-off value for determination of mean geometric titers.

2.3. Determination of the avidity of HBsAg-specific antibodies

The avidities of IgG1 and IgG3 isotypes of anti-HBs were determined by an ELISA elution assay using thiocyanate (NH_4SCN) as a chaotropic agent, according to a well established method [28–31] that was previously standardized in our laboratory for a panel of protein antigens [32] and which was specifically adapted to HBsAg-specific Ab. This method has been previously shown [29] to correlate with a more direct measurement of affinity. It provides a convenient approach for the estimation of human polyclonal antibody avidity in conditions where direct assays are hardly applicable. Assay validation included the assessment of antigen stability following incubation with up to 6 M NH_4SCN and the definition of optimal temperature and duration of incubation periods. Sera were pre-titered and adjusted by dilution to a concentration giving ELISA results in the upper portion of the linear part of the standard curve (OD approximately 1.5). Following incubation of plasma dilutions, NH_4SCN was added at a final concentration ranging from 0 to 4 M. The plates were then incubated for 15 min at room temperature, prior to the addition of goat anti-human IgG1/IgG3 antibodies and processing as described above. The amount of antibody remaining bound to the plate, at each NH_4SCN concentration, was calculated in units by reference to the ELISA standard curve. The avidity index (AI), corresponding to the concentration of thiocyanate required to elute 50% of the Ab units, was calculated as described previously [29]. The avidity profile was determined by calculation of the amount of Ab eluted for each increase in NH_4SCN concentration. Given the poor reproducibility of elution analyses for sera whose OD did not reach a minimal value of 1.0 at a 1/50 dilution, we decided to consider these values as not interpretable (NI). Low avidity Ab were defined by the fraction eluted at <1 M NH_4SCN concentrations, whereas high-avidity IgG1 Ab were defined as those only eluted at ≥2 M NH_4SCN concentrations. Medium avidity was Ab eluted between >1 and 2 M of NH_4SCN.

2.4. Statistical analyses

All efficacy analyses were conducted according to the intent-to-treat/attrition adjusted population with the use of two-tailed tests and a type I error of 0.05. Two subjects did not complete the full course of vaccination. In these cases, immunogenicity data were included for time points up to the time of missing doses but not after. Missing data were not replaced but were treated as missing. The following efficacy parameters were summarized and presented: (i) summary of anti-HBs immunoglobulin isotypes (geometric mean of IgG1 and IgG3 titers); (ii) summary of anti-HBs avidity index; (iii) summary of anti-HBs avidity (proportion of subject with low and high avidity). Comparison of control group (Engerix-B®) with single or combined experimental groups (Engerix-B® + CpG 7909 (0.5 mg), and/or Engerix-B® + CpG 7909 (1.0 mg), and/or Engerix-B® + CpG 7909 (0.125 mg)) were performed using *t*-test for continuous variable and Chi-square for dichotomous data. These analyses were performed using SAS® version 8.2. Statistical analysis between IgG/IgG1/IgG3 titers obtained in various groups was performed using the Mann–Whitney *U*–Wilcoxon Rank sum *W* test with SPSS 11.5 for Windows. Differences with probability values >0.05 were considered insignificant.

3. Results

3.1. Influence of CpG 7909 on avidity maturation of anti-HBs IgG1 response

Immunization with alum-adjuvanted HBV vaccine (Engerix-B®) whether given on its own (EngB) or with added CpG 7909 (EngB-CpG), resulted in the preferential induction of IgG1 anti-HBs, with significantly weaker IgG3 and little or no IgG2 and IgG4 Ab (not shown). We studied the Ag-specific avidity of IgG1 and IgG3 anti-HBs at weeks 0, 8, 26 and 52 after priming. As expected, no subjects had detectable anti-HBs at week 0 since seronegativity for HBV antigens had been an inclusion criterion. For all subsequent time-points, anti-HBs IgG1 were significantly higher in subjects immunized with Eng-B plus 0.5 mg of CpG 7909 compared to controls receiving Eng-B alone (Fig. 1A). In controls, anti-HBs IgG1 titers did not reach levels sufficient for reliable avidity analyses until after all three vaccine doses were given. Thus for control subjects it was possible to determine avidity index (AI) for only two time points, 26 and 52 weeks (Fig. 1B). In subjects receiving the vaccine with CpG 7909 (0.5 mg), a progressive increase of AI was observed over time, reflecting the progression of Ab avidity during the first 6 months after priming and with additional vaccine doses (Fig. 1B). AI remained similar between weeks 26 and 52 in each group, suggesting that the avidity maturation process was essentially completed by week 26. Thus, CpG 7909 treatment elicited anti-HBs IgG1 with significantly higher avidity than those in control subjects ($P < 0.01$), both at the end of the series of three doses, and 6 months later, which is 1 year after priming (Fig. 1B).

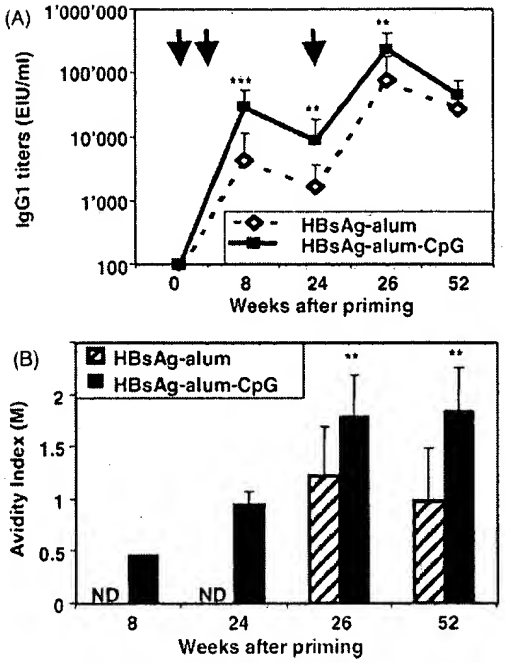

Fig. 1. CpG 7909 enhances titers and avidity of anti-HBsAg IgG1 antibodies. Anti-HBsAg IgG1 titers (A) and avidity indexes (B) were assessed by ELISA, as described in Section 2, at various intervals after immunization of healthy human adults with alum-adsorbed HBsAg alone or + CpG 7909 (0.5 mg). Arrows indicate timing of immunization. ND, not determinable. Statistical significance: $^{**}P < 0.01$, $^{***}P < 0.001$.

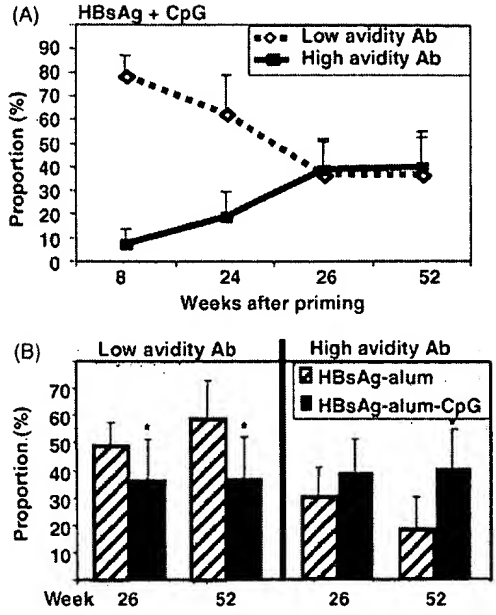

Fig. 2. Distribution of low- and high-avidity IgG1 anti-HBsAg Ab following immunization with HBsAg alone or with CpG 7909. The proportion of low- and high-avidity IgG1 anti-HBsAg was assessed as described in Section 2, at various intervals after immunization with HBsAg + CpG 7909 (0.5 mg) (A). Comparison at weeks 26 and 52 among individuals immunized with HBsAg alone or + CpG 7909 (0.5 mg) demonstrates significant differences ($P < 0.05$) between the proportion of low- and high-avidity IgG1 Ab in both groups (B).

3.2. *Influence of CpG 7909 on the avidity distribution of anti-HBs IgG1 antibodies*

Avidity indexes, which are most frequently used as markers of avidity, may mask differences in avidity profile if there is only a small pool of high- or low-avidity antibodies. The relative persistence of low avidity antibodies or the rate of appearance of higher avidity antibodies can be better appreciated by calculating the percentage of Ab molecules that can be dissociated at given concentrations of chaotropic ions. The incremental Ab release from solid-phase HBsAg in the presence of increasing concentrations of NH_4SCN was analyzed in detail. This allowed for an indirect visualization of the dynamics of Ab populations of differing avidity at both early and late time points after immunization. At 8 weeks post priming with HBsAg + CpG 7909 (0.5 mg), most (78.6%) IgG1 Ab were still of low avidity (eluted at <1 M NH_4SCN concentrations), whereas higher avidity IgG1 Ab (eluted at over 2 M NH_4SCN concentrations) represented less than 10% of total HBsAg-specific IgG1 Ab (Fig. 2A). Thus, high-avidity Ab were rare at this 8-week time-point, despite previous administration of two doses of EngB-CpG. Over time, there was a gradual decline of low-avidity Ab and a concomitant increase of high-avidity Ab. Significant changes in the distribution of low- and high-avidity Ab were observed between each consecutive visit from weeks 8 to 26 (Fig. 2A). The addition of CpG 7909 to EngB significantly enhanced the proportion of high-avidity IgG1 anti-HBs both at the end of the primary series (week 26) and 1 year after priming (Fig. 2B). One should note that the strongest relative change in the distribution of low- and high-avidity Ab between two consecutive visits was observed between weeks 24 and 26, i.e. immediately prior to and 2 weeks after the administration of the third vaccine dose. In contrast, there was little change between 26 and 52 weeks when no additional vaccine doses were given. The apparent increase of low-avidity in control individuals that received only EngB at week 52 compared to week 26 (Fig. 2B) did not reach statistical significance. During the same period, the proportion of high-avidity Ab clearly remained stable in the group receiving CpG 7909.

3.3. *Influence of CpG 7909 adjuvant on the avidity profile of anti-HBs IgG3 antibodies*

Immunization with EngB also induces IgG3 anti-HBs, although at markedly lower titers than for IgG1 (Fig. 3A). Addition of CpG 7909 (0.5 mg) enhanced anti-HBs IgG3 titers, which reached five-fold higher levels at week 26 than those in controls (Fig. 3A). Avidity analyses were done on sera from a subset of subjects with sufficient levels of anti-HBs IgG3 titers to allow for reliable testing at each time point. Surprisingly, the enhancement of anti-HBs IgG3 responses by CpG was not associated with significant changes in avidity indexes (Fig. 3B) or in the distribution of low-avidity (mean 31.7%

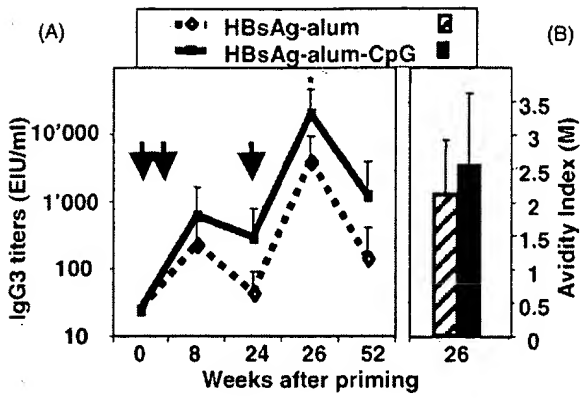

Fig. 3. CpG 7909 enhances IgG3 anti-HBsAg titers but not avidity. Anti-HBsAg IgG3 titers (A) and avidity indexes (B) were assessed by ELISA as described, at various intervals after immunization of healthy human adults with alum-adsorbed HBsAg alone or + CpG 7909 (0.5 mg). Arrows indicate timing of immunization. Statistical significance: $P < 0.05$.

with CpG 7909 versus 34.3% in controls) or high-avidity Ab at week 26 (50.7% versus 47.3%).

3.4. Influence of the dose of CpG 7909 on the avidity of anti-HBs IgG1 antibodies

We next compared the influence of three different doses of CpG 7909 (0.125, 0.5 and 1 mg) on the avidity maturation process of anti-HBs IgG1. At weeks 8, 24 and 26, the two highest doses (0.5 and 1 mg) of CpG 7909 elicited significantly higher anti-HBs IgG1 than did the lower (0.125 mg) dose (Fig. 4A). Avidity indexes progressively increased from weeks 8 to 26, reaching significantly higher mean values at 26 and 52 weeks in the 3 CpG 7909-treated groups than in controls ($P < 0.001$, Fig. 4B). The AI in subjects receiving the highest dose of CpG 7909 (1 mg), was significantly higher at the end of the primary series (week 26) than that in subjects receiving 0.5 mg ($P < 0.001$) or 0.125 mg ($P < 0.01$) CpG 7909, or control subjects receiving no CpG 7909 ($P < 0.0001$) (Fig. 4B). Although the lowest dose of CpG 7909 (0.125 mg) did not significantly enhance IgG1 titers compared to those in controls (Fig. 4A), it was nevertheless sufficient to induce IgG1 Ab of a significantly higher AI ($P < 0.01$, Fig. 4B). This was reflected by a markedly higher proportion of high-avidity Ab (44% at week 52 compared to 18% in controls, $P < 0.05$). This prompted us to analyze possible correlations between individual anti-HBs IgG1 and AI at 26 weeks. Importantly, the avidity of IgG1 anti-HBs elicited by immunization with Engerix-B, whether administered alone or with added CpG 7909, could not be predicted on the basis of titers of circulating anti-HBsAg. The level of correlation observed following immunization was fairly strong in the control group receiving EngB alone ($R^2 = 0.7126$), but much less in the EngB-CpG group ($R^2 = 0.254$, 0.0523, and 0.0691 for 0.125, 0.5 and 1.0 mg CpG 7909, respectively). Thus, CpG 7909 may exert a distinct influence on the IgG1

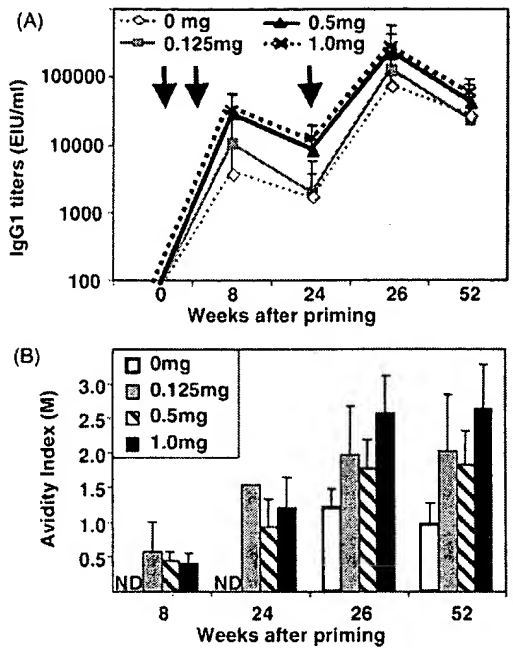

Fig. 4. Influence of the dose of CpG 7909 on IgG1 anti-HBsAg. Anti-HBsAg IgG1 titers (A) and avidity indexes (B) were assessed by ELISA at various intervals after immunization with alum-adsorbed HBsAg alone or with indicated doses of CpG 7909. Arrows indicate timing of immunization. ND, not determinable. Significant differences are indicated in Section 3.

production and on the avidity maturation of HBsAg-specific B cells.

3.5. Influence of CpG 7909 on the avidity of anti-TT IgG antibodies

It was recently hypothesized that CpG oligos behaved as polyclonal activators which activate memory B cells regardless of their Ag-specificity [33]. This hypothesis was tested here by assessing circulating Ab levels to tetanus toxoid (TT) before and after administration of EngB with or without added CpG 7909. TT-specific Ab were present in all individuals at study initiation, ranging from very low (69 mIE/ml) to high (7198 mIE/ml) values depending on previous immunizations. As polyclonal activation could possibly result into only transiently increased Ab, analyses compared TT Ab at 24 and 26 weeks, i.e. before and 2 weeks after administration of HBsAg alone or + CpG 7909 (0.5 mg). TT-specific titers remained similar in each individual (Fig. 5), as confirmed by the mean post/pre-injection ratio (0.91 ± 0.14 following injection of CpG 7909 and 0.97 ± 0.15 in controls). Similar results were obtained when comparing anti-TT titers at weeks 0 and 8, i.e. before and 1 month after the 2nd injection of CpG 7909, or in individuals injected with 1 mg of CpG 7909 (not shown). Thus, despite prior TT immunization, administration of the CpG 7909 adjuvanted vaccine did not lead to an increase production of TT-specific Ab. This may indicate a lack of effect on TT-specific memory B cells in these conditions of immunization.

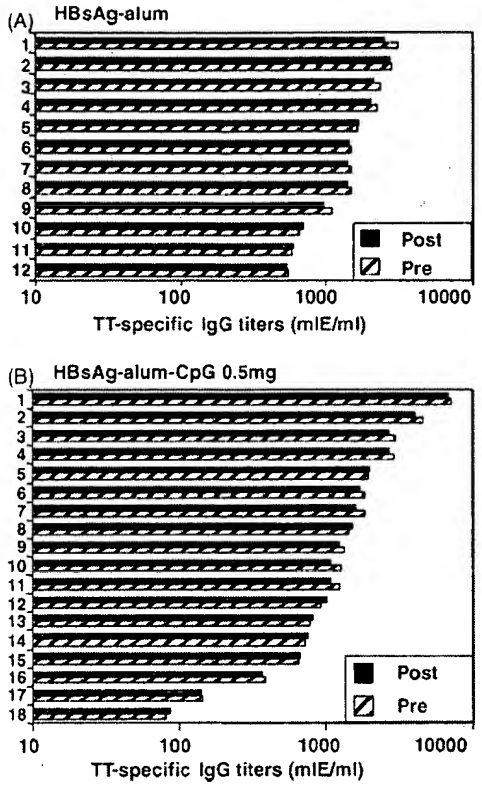

Fig. 5. Administration of CpG 7909 does not increase anti-TT antibodies. Anti-TT IgG titers were assessed by ELISA before (pre, week 24) and 2 weeks after (post, week 26) administration of alum-adsorbed HBsAg alone (A) or + CpG 7909 (0.5 mg) (B). Anti-TT titers measured in paired plasma samples are represented for all individuals of group A (n = 12) and B (n = 18).

4. Discussion

This study demonstrates that the addition of CpG 7909 to an HBV vaccine containing alum-adsorbed HBsAg leads not only to a significant increase of human anti-HBs Ab titers but as well to a significant increase of anti-HBs IgG1 antibody avidity. This qualitative influence appears during a late phase of the B cell response, i.e. between 2 and 6 months after priming, and it probably reflects an enhancement of the late avidity maturation process.

Avidity maturation is thought to result from B cell proliferation, GC differentiation, somatic hypermutation in the V(D)H region and clonal competition for antigen. CpG oligos could act at several stages of this B cell differentiation process. First, they could enhance the recruitment of naïve human B cells into the response and their differentiation into ASC by providing additional activation and proliferation signals to B cells, as they do in vitro [34]. However, the magnitude of the IgG response does not correlate with the number of Ag-specific B cells initially recruited into the response [35], and the enhancement of both anti-HBs IgG1 titers and avidity for antigen implies that CpG oligos may also exert their influence at subsequent stages of the B cell differentiation process.

It is known that during the GC reaction, Ag-specific B cells rapidly expand and diversify their antigen receptors through somatic hypermutation and receptor editing. While the vast majority of these essentially random changes are deleterious to antigen binding, some mutations increase the cell's affinity for antigen and result in their positive selection (reviewed in [36]). Thus, CpG oligos could influence occurrence of somatic mutations early in the GC reaction, B cell selection within the GC and/or differentiation of GC B cells into ASC. Potential targets for a CpG-mediated influence during the GC reaction include B cells themselves, which express TLR-9 after B cell receptor (BCR) triggering [34,37], but also pDC which could increase GC B cell expansion and ASC differentiation both directly via enhanced IL-12 production [38] and indirectly via enhanced activation of $CD4^+$ GC T cells. The marked enhancement of IgG1 anti-HBs titers 8 weeks after priming suggests that CpG oligos do enhance GC B cell differentiation into IgG1-secreting plasma cells, as observed in vitro [34]. However, at this time point, low-avidity Ab are still represent more than 80% of HBsAg-specific IgG1 Ab. This fact argues against a direct influence of CpG oligos on somatic hypermutation.

Although the duration of Ag-induced GC is unknown in humans, animal studies have indicated that the GC reaction does not exceed a few weeks. Therefore, the observed increase of the proportion of high-avidity Ab between 8 and 26 weeks after priming may reflect an influence of CpG oligos on a late maturation process that is probably occurring in a post-GC period. Data obtained in mice have recently indicated that although the generation of high-affinity B cells is dependent on the GC reaction, the selection for higher affinity B cells continues into the bone marrow long after the end of the GC reaction. This is mirrored by the progressive increase of avidity of circulating Ab [12]. In our study, the selection process appears to be completed by 26 weeks after priming. since there was no further change in avidity profile between weeks 26 and 52. At 52 weeks after priming, Ab are presumably produced by long-lived ASC residing in the bone marrow.

Exactly where and how this influence of CpG oligos takes place remains to be defined. Our recent studies in mice indicated that addition of CpG oligos to a single dose of alum-adsorbed tetanus toxoid (TT) markedly enhanced TT-specific ASC splenic responses and prolonged their duration by several months, resulting into a significantly increased BM pool of TT-specific ASC [39]. This occurred without detectable influence on TT-induced GC numbers or size. The prolonged persistence of ASC in a post-GC splenic compartment could result from an anti-apoptotic effect of CpG oligos. It is known that CpG oligos do increase B cell mRNA expression of egr-1, c-jun, c-myc and bcl-x_L (reviewed in [15]), and drive them into G1 and S phase [17]. CpG oligos almost completely prevent spontaneous murine or human B cell apoptosis in vitro. An alternative hypothesis is that CpG oligos may increase ASC survival indirectly, by preventing apoptosis of specialized cells important for post-GC ASC survival in the spleen or bone marrow.

As CpG oligos essentially influence avidity maturation late in the B cell response, it was important to define whether they also influence induction of memory B cells. Previous studies performed in vitro did not suggest that activation with CpG oligos had an effect on memory B cell generation [34]. However, they did show an enhanced proliferation and ASC differentiation of existing human memory B cells [33]. There was a marked change of the avidity profile towards higher-avidity IgG1 Ab between weeks 24 and 26, i.e. immediately prior and 2 weeks after the third vaccine dose. This suggests that CpG oligos may favor the accumulation of higher-affinity B cells in the resting memory B cell pool that could be rapidly reactivated by the third vaccine dose. Recent in vitro and in vivo studies indicated that CpG oligos can act as polyclonal activators for memory B cells [33]. In our study, the local administration of CpG 7909 did not appear to influence the level of pre-existing anti-TT antibodies. This may indicate that synergistic activation through the BCR is by far more efficient for memory B cell reactivation than the limited polyclonal activation that can be induced at the regional lymph node level by administration of a CpG containing vaccine.

An obvious question is whether the long lasting production of higher-avidity anti-HBs antibodies and of higher-avidity memory B cells may provide additional clinical benefit. Germ-line Ab directed against certain pathogens expressing repetitive Ab determinants have occasionally been shown to have neutralization properties similar to those of hypermutated Ab [40]. However, in many other conditions, a strong correlation was seen between avidity and viral neutralization capacity [41–44]. For example, high-avidity antibodies could confer a 100% protective efficacy in a model of measles encephalitis whereas only 50% efficacy was obtained with lower-avidity Ab [45]. Mechanisms of HBV neutralization are yet poorly understood. Antibodies of higher Ag-binding avidity were shown to have a more potent neutralizing activity in primary hepatocyte culture [46]. Therefore, it is conceivable that a higher neutralization capacity would confer better protection when only limited levels of antibodies are persisting, i.e. late after immunization. Enhanced avidity maturation following HBV vaccination may also represent a significant advantage when viral exposure occurs after disappearance of circulating Ab. At this stage, protection requires memory B cells to be activated and to produce Ab capable of viral neutralization. The existence of higher-avidity memory B cells should result in a more rapid and efficient curtailment of the infectious process, thereby reducing the duration of acute infection and period of contagion.

This study is the first demonstration that a novel adjuvant can enhance avidity maturation in humans and possibly increase the viral neutralization capacity. Such enhancement may be isotype-specific and it cannot be predicted on the basis of antibody titers. These results suggest that it would be worthwhile to assess all novel adjuvants for their capacity to qualitatively influence the B cell differentiation process. These observations may have important implications for the design of new vaccines.

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References

[1] Jacob J, Kelsoe G. In situ studies of the primary immune response to (4-hydroxy-3-nitrophenyl)acetyl. II. A common clonal origin for periarteriolar lymphoid sheath-associated foci and germinal centers. J Exp Med 1992;176(3):679–87.
[2] McHeyzer-Williams MG, McLean MJ, Lalor PA, Nossal GJ. Antigen-driven B cell differentiation in vivo. J Exp Med 1993;178(1):295–307.
[3] Griffiths GM, Berek C, Kaartinen M, Milstein C. Somatic mutation and the maturation of immune response to 2-phenyl oxazolone. Nature 1984;312(5991):271–5.
[4] French DL, Laskov R, Scharff MD. The role of somatic hypermutation in the generation of antibody diversity. Science 1989;244(4909):1152–7.
[5] Siskind GW, Benacerraf B. Cell selection by antigen in the immune response. Adv Immunol 1969;10:1–50.
[6] Jacob J, Kelsoe G, Rajewsky K, Weiss U. Intraclonal generation of antibody mutants in germinal centres. Nature 1991;354(6352):389–92.
[7] Berek C, Berger A, Apel M. Maturation of the immune response in germinal centers. Cell 1991;67(6):1121–9.
[8] Kelsoe G. Life and death in germinal centers (redux). Immunity 1996;4(2):107–11.
[9] Berek C, Griffiths GM, Milstein C. Molecular events during maturation of the immune response to oxazolone. Nature 1985;316(6027):412–8.
[10] Cumano A, Rajewsky K. Clonal recruitment and somatic mutation in the generation of immunological memory to the hapten NP. EMBO J 1986;5(10):2459–68.
[11] Foote J, Milstein C. Kinetic maturation of an immune response. Nature 1991;352(6335):530–2.
[12] Takahashi Y, Dutta PR, Cerasoli DM, Kelsoe G. In situ studies of the primary immune response to (4-hydroxy-3-nitrophenyl)acetyl. V. Affinity maturation develops in two stages of clonal selection. J Exp Med 1998;187(6):885–95.
[13] Karush F. Comprehensive immunology. New York: Plenum; 1978.
[14] Krieg AM. Immune effects and mechanisms of action of CpG motifs. Vaccine 2000;19(6):618–22.
[15] Krieg AM, Yi AK. Rescue of B cells from apoptosis by immune stimulatory CpG DNA. Springer Semin Immunopathol 2000;22(1/2):55–61.
[16] Yi AK, Peckham DW, Ashman RF, Krieg AM. CpG DNA rescues B cells from apoptosis by activating NFkappaB and preventing mito-

chondrial membrane potential disruption via a chloroquine-sensitive pathway. Int Immunol 1999;11(12):2015–24.
[17] Yi AK, Chang M, Peckham DW, Krieg AM, Ashman RF. CpG oligodeoxyribonucleotides rescue mature spleen B cells from spontaneous apoptosis and promote cell cycle entry. J Immunol 1998;160(12):5898–906.
[18] Yi AK, Hornbeck P, Lafrenz DE, Krieg AM. CpG DNA rescue of murine B lymphoma cells from anti-IgM-induced growth arrest and programmed cell death is associated with increased expression of c-myc and bcl-xL. J Immunol 1996;157(11):4918–25.
[19] Jahrsdorfer B, Hartmann G, Racila E, Jackson W, Muhlenhoff L, Meinhardt G, et al. CpG DNA increases primary malignant B cell expression of costimulatory molecules and target antigens. J Leukoc Biol 2001;69(1):81–8.
[20] Krieg AM, Yi AK, Matson S, Waldschmidt TJ, Bishop GA, Teasdale R, et al. CpG motifs in bacterial DNA trigger direct B-cell activation. Nature 1995;374(6522):546–9.
[21] Klinman DM, Barnhart KM, Conover J. CpG motifs as immune adjuvants. Vaccine 1999;17(1):19–25.
[22] Krieg AM, Davis HL. Enhancing vaccines with immune stimulatory CpG DNA. Curr Opin Mol Ther 2001;3(1):15–24.
[23] Brazolot Millan CL, Weeratna R, Krieg AM, Siegrist CA, Davis HL. CpG DNA can induce strong Th1 humoral and cell-mediated immune responses against hepatitis B surface antigen in young mice. Proc Natl Acad Sci USA 1998;95(26):15553–8.
[24] Kovarik J, Bozzotti P, Love-Homan L, Pihlgren M, Davis HL, Lambert PH, et al. CpG oligodeoxynucleotides can circumvent the Th2 polarization of neonatal responses to vaccines but may fail to fully redirect Th2 responses established by neonatal priming. J Immunol 1999;162(3):1611–7.
[25] Kovarik J, Bozzotti P, Tougne C, Davis HL, Lambert PH, Krieg AM, et al. Adjuvant effects of CpG oligodeoxynucleotides on responses against T-independent type 2 antigens. Immunology 2001;102(1):67–76.
[26] Krieg AM. CpG motifs in bacterial DNA and their immune effects. Annu Rev Immunol 2002;20:709–60.
[27] Horner AA, Raz E. Immunostimulatory sequence oligodeoxynucleotide-based vaccination and immunomodulation: two unique but complementary strategies for the treatment of allergic diseases. J Allergy Clin Immunol 2002;110(5):706–12.
[28] Macdonald RA, Hosking CS, Jones CL. The measurement of relative antibody affinity by ELISA using thiocyanate elution. J Immunol Methods 1988;106(2):191–4.
[29] Goldblatt D. Simple solid phase assays of avidity. In: Turner M, Johnstone A, editors. Immunochemistry. 2. A practical approach. Oxford: IRL Press at Oxford University Press; 1997. p. 31–51.
[30] McCloskey N, Turner MW, Goldblatt TD. Correlation between the avidity of mouse-human chimeric IgG subclass monoclonal antibodies measured by solid-phase elution ELISA and biospecific interaction analysis (BIA). J Immunol Methods 1997;205(1):67–72.
[31] Goldblatt D, Vaz AR, Miller E. Antibody avidity as a surrogate marker of successful priming by *Haemophilus influenzae* type b conjugate vaccines following infant immunization. J Infect Dis 1998;177(4):1112–5.
[32] Schallert N, Pihlgren M, Kovarik J, Roduit C, Tougne C, Bozzotti P, et al. Generation of adult-like antibody avidity profiles after early-life immunization with protein vaccines. Eur J Immunol 2002;32(3):752–60.
[33] Bernasconi NL, Traggiai E, Lanzavecchia A. Maintenance of serological memory by polyclonal activation of human memory B cells. Science 2002;298(5601):2199–202.
[34] Jung J, Yi AK, Zhang X, Choe J, Li L, Choi YS. Distinct response of human B cell subpopulations in recognition of an innate immune signal CpG DNA. J Immunol 2002;169(5):2368–73.
[35] Shih TA, Meffre E, Roederer M, Nussenzweig MC. Role of BCR affinity in T cell dependent antibody responses in vivo. Nat Immunol 2002;3(6):570–5.
[36] McHeyzer-Williams LJ, Driver DJ, McHeyzer-Williams MG. Germinal center reaction. Curr Opin Hematol 2001;8(1):52–9.
[37] Bernasconi NL, Onai N, Lanzavecchia A. A role for Toll-like receptors in acquired immunity: upregulation of TLR9 by BCR triggering in naive B cells and constitutive expression in memory B cells. Blood 2003;30:30.
[38] Dubois B, Barthelemy C, Durand I, Liu YJ, Caux C, Briere F. Toward a role of dendritic cells in the germinal center reaction: triggering of B cell proliferation and isotype switching. J Immunol 1999;162(6):3428–36.
[39] Pihlgren M, Tougne C, Schallert N, Bozzotti P, Lambert PH, Siegrist CA. CpG-motifs enhance initial and sustained primary tetanus-specific antibody secreting cell responses in spleen and bone marrow, but are more effective in adult than in neonatal mice. Vaccine 2003;21(19/20):2492–9.
[40] Kalinke U, Oxenius A, Lopez-Macias C, Zinkernagel RM, Hengartner H. Virus neutralization by germ-line vs. hypermutated antibodies. Proc Natl Acad Sci USA 2000;97(18):10126–31.
[41] Toran JL, Sanchez-Pulido L, Kremer L, del Real G, Valencia A, Martinez AC. Improvement in affinity and HIV-1 neutralization by somatic mutation in the heavy chain first complementarity-determining region of antibodies triggered by HIV-1 infection. Eur J Immunol 2001;31(1):128–37.
[42] Yuan W, Parrish CR. Comparison of two single-chain antibodies that neutralize canine parvovirus: analysis of an antibody-combining site and mechanisms of neutralization. Virology 2000;269(2):471–80.
[43] Kostolansky F, Vareckova E, Betakova T, Mucha V, Russ G, Wharton SA. The strong positive correlation between effective affinity and infectivity neutralization of highly cross-reactive monoclonal antibody IIB4, which recognizes antigenic site B on influenza A virus haemagglutinin. J Gen Virol 2000;81(Pt 7):1727–35.
[44] Lantto J, Lindroth Y, Ohlin M. Non-germ-line encoded residues are critical for effective antibody recognition of a poorly immunogenic neutralization epitope on glycoprotein B of human cytomegalovirus. Eur J Immunol 2002;32(6):1659–69.
[45] Olszewska W, Obeid OE, Steward MW. Protection against measles virus-induced encephalitis by anti-mimotope antibodies: the role of antibody affinity. Virology 2000;272(1):98–105.
[46] Ryu CJ, Gripon P, Park HR, Park SS, Kim YK, Guguen-Guillouzo C, et al. In vitro neutralization of hepatitis B virus by monoclonal antibodies against the viral surface antigen. J Med Virol 1997;52(2):226–33.

Selective immune redirection in humans with ragweed allergy by injecting Amb a 1 linked to immunostimulatory DNA

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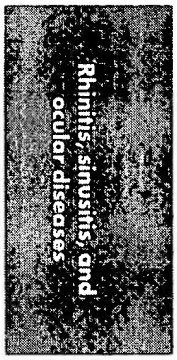

Background: In animal models administration of immunostimulatory DNA sequences preferentially elicits T_H1-dominated (type 1-dominated) immunity and can inhibit developing or ongoing T_H2 (type 2) responses.

Objective: Our objective was to investigate this phenomenon in humans.

Methods: In a randomized, third party-blinded, placebo-controlled, proof-of-concept study conducted entirely in the winter in 19 adults with ragweed allergy, we administered 6 subcutaneous injections of purified Amb a 1 linked to the 22-base-long immunostimulatory phosphorothioate oligodeoxyribonucleotide 1018 (Amb a 1–immunostimulatory DNA sequence conjugate [AIC]). Before the course of AIC or placebo injections and 2 and 16 weeks afterward, we measured recall responses to ragweed, streptokinase, and PHA in short-term primary culture of fresh PBMCs after restimulation with antigen. We quantified regulatory cytokine and chemokine responses characteristic of T_H2 immunity (IL-5, IL-13, CCL17 [TARC], and CCL22 [MDC]), and T_H1 immunity (IFN-γ, CXCL9 [Mig], and CXCL10 [IP-10]), as well as IL-10, a cytokine sometimes linked to regulatory T-cell populations.

Results: We demonstrated for the first time that human systemic in vivo ragweed-specific T_H2 responses were selectively redirected toward T_H1 responses, with significant increases in IFN-γ, CXCL9, and CXCL10 and significant decreases in IL-5, CCL17, and CCL22 found at 2 and 16 weeks after the sixth injection. Cytokine and chemokine responses to the unrelated bacterial antigen streptokinase and the global capacity to mount immune responses on polyclonal activation with PHA did not change. No clinically significant systemic or local allergic reactions were associated with AIC or placebo injections.

Conclusions: AIC, injected in concentrations that were approximately 40-fold lower than those used in most murine studies published to date, led to a prolonged shift from T_H2 immunity toward T_H1 immunity and appeared to be safe. This novel approach has the potential for immune redirection in human immediate hypersensitivity diseases. (J Allergy Clin Immunol 2004;113:1144-51.)

Key words: Allergen-specific immunotherapy, allergen vaccines, Amb a 1, chemokines, cytokines, DNA vaccines, humans, immunostimulatory DNA sequences, ragweed allergy

Redirecting the underlying immune dysfunction that elicits and maintains immediate hypersensitivity diseases in humans remains an unmet challenge.[1] The immunologic mechanisms associated with these diseases involve a spectrum of $CD4^+$ T cells, including regulatory T cells, $CD25^+$ cells, natural killer T cells, and T_H1, T_H2, and T_H3 cells.[2] Allergen-specific immunotherapy aims to correct this underlying immune imbalance, particularly by attempting to redirect the established T_H2-dominated response to a more balanced T_H1/T_H2 response.[3-6] Although conventional allergen-specific immunotherapy has proved dose-related efficacy, it is neither optimally convenient nor perfectly safe. Considerable attention has therefore been focused on development of novel immunotherapy strategies.[7,8]

In animal studies immunostimulatory DNA sequences (ISSs), which occur naturally in prokaryotes but are largely absent from vertebrates, induce T_H1-biased (type 1-biased) immune responses and inhibit development of T_H2 (type 2) immunity when administered before antigen exposure,[9-13] thus effectively inducing a strong and long-lasting commitment to T_H1-biased immunoregulatory responses. ISS administration to animals with established immediate hypersensitivity, in which the allergen-specific response is already committed to a T_H2 phenotype, has been less consistently effective. Although some investigators have had little success in redirecting pre-established T_H2-dominated immunity,[14,15] others[16,17] have elicited 2- to 4-fold reductions in T_H2 cytokines, such as IL-5, concomitant with similar increases in IFN-γ, suggesting that DNA-based therapeutics are potentially useful for the treatment of human immediate hypersensitivity diseases.[18,19]

Amb a 1 predominates among approximately 20 allergens in *Ambrosia artemisiifolia*. It has a molecular mass of 37,800 d, has been sequenced and otherwise thoroughly studied, and is currently used to standardize short ragweed allergen extracts and to define the target dose of short ragweed allergen in conventional immunotherapy.[7]

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Abbreviations used
Ag: Antigen
AIC: Amb a 1–immunostimulatory DNA sequence conjugate
Amb a 1: Immunodominant allergen from *Ambrosia artemisiifolia* (short ragweed)
ANA: Anti-nuclear antibody
ANOVA: Analysis of variance
C: Complement
CD: Cluster of differentiation
DNA: Deoxyribonucleic acid
ELISA: Enzyme-linked immunosorbent assay
IFN-γ: Interferon-gamma
IgE: Immunoglobulin E
IL: Interleukin
ISS: Immunostimulatory DNA sequence
MHC: Major histocompatibility complex
PBS: Phosphate-buffered saline
PHA: Phytohemagglutinin
SE: Standard error
T_H: T helper

Preclinical rodent studies have demonstrated that conjugation of Amb a 1 and ISS leads to more potent induction of Amb a 1–specific T_H1 immune responses and greater suppression of T_H2 immune responses than a simple mixture of Amb a 1 and ISS.[20] The effect of ISSs added to cultures of human cells suggests their potential for revolutionizing allergen-specific immunologic treatment.[11,19-24] We hypothesized that Amb a 1 linked to ISS would have the capacity to redirect pre-established allergen-specific immunoregulatory cytokine and chemokine recall responses in humans with ragweed allergy.

METHODS

This randomized, third party–blinded, placebo-controlled Phase I investigation was approved by the University of Manitoba Research Ethics Board, and written informed consent was obtained from all participants. The study was conducted entirely in midwinter, when there was no exposure to ragweed or other pollens. Individuals received 6 injections of investigational vaccine or placebo administered at weekly intervals (Fig 1).

Investigational vaccine

The Amb a 1 immunostimulatory DNA sequences conjugate (AIC) vaccine consisted of purified Amb a 1 linked to the immunostimulatory phosphorothioate oligodeoxyribonucleotide 1018 (22 bases in length with sequence of 5′-TGACTGTG-AACGTTCGAGATGA-3′; prepared at Avecia, Boston, Mass, for Dynavax Technologies Corp, Berkeley, Calif). Each vial contained 0.7 mL of AIC at a concentration of 30 μg/mL by protein in PBS. Thirty micrograms per milliliter of AIC contained approximately 28 μg/mL ISS 1018. The AIC was stored at −60°C, thawed at room temperature, and diluted serially with PBS. PBS was also used for control injections. Throughout the study, all participants and clinical and laboratory personnel involved were blinded as to whether AIC or placebo was injected, with the exception of one nurse who prepared and injected the AIC or placebo but had no other involvement.

Participants

We included individuals age 18 to 60 years who had a history of fall allergic rhinitis symptoms and a positive epicutaneous test result of at least Σ_{10} (sum of longest diameter of erythema plus width of erythema measured perpendicularly to longest diameter) to licensed standardized ragweed extract (Greer Laboratories, Inc, Lenoir, NC). Individuals were excluded if they had any clinically significant illness, were pregnant, were breast-feeding, or required daily treatment for asthma at the time of the study; had ever had a hospital admission for asthma or received anti-IgE antibody; had received ragweed immunotherapy within the previous 5 years; or had taken immunosuppressive medication, including systemic corticosteroids, within the previous month. Throughout the study, AIC or placebo injections were administered only if participants continued to meet these criteria. Women of child-bearing potential had negative pregnancy test results at study entry and before each injection.

Study plan

At baseline before the first subcutaneous injection of AIC or placebo and 2 and 16 weeks after the sixth and last injection, medical history was taken, physical examination was performed, and 40 mL of blood was obtained for immunologic tests (Fig 1). Weekly AIC or placebo injections in incremental doses of 0.06, 0.3, 1.2, 3.0, 6.0, and 12.0 μg were scheduled. Adjustments in the dose regimen were made for missed injections and for local or systemic reactions to injections, if any. The AIC or placebo dose was increased if the preceding injection resulted in a local reaction of less than 2 cm in mean diameter, repeated if the injection resulted in a local reaction of 2 to 4 cm in mean diameter, and decreased by 50% if a local reaction of greater than 4 cm in mean diameter or a systemic reaction occurred. Participants who experienced a second reaction on dose escalation (following the 50% dose reduction after their first reaction) had their dose decreased by 50% and subsequently received the reduced dose until the end of the study.

Immunologic tests

Fresh PBMCs were isolated and used for short-term primary culture at 5×10^6/mL for antigen and 2.5×10^6/mL for polyclonal stimulation, with at least 2 wells per condition. Cells were cultured (1) in the absence of stimuli; (2) with standardized short ragweed extract (309 antigen units/mL, 25 mg/mL; stock from Greer Laboratories Inc, Lenoir, NC), titrating each subject over a concentration range of 1, 10, and 100 μg/mL; and (3) with recall antigen streptokinase (Aventis Pharma, Montreal, Quebec, Canada) at 5000 U/mL or PHA (Sigma-Aldrich Canada Ltd, Oakville, Ontario, Canada) at suboptimal (2 μg/mL) and optimal (10 μg/mL) concentrations. Culture supernatants were harvested at the times we identified in previous experiments to yield optimal cytokine and chemokine responses.[25-27] Standardized short ragweed extract, rather than Amb a 1, was used as the stimulating antigen because it is readily available, involves a broader panel of antigens than Amb a 1, and, as the extract widely used in skin testing and conventional immunotherapy, is highly clinically relevant.[7]

Allergen-driven and polyclonally stimulated T_H2 immunity-associated IL-4, IL-5, IL-13, and the chemokines CCL1 (I-309), CCL2 (MCP-1), CCL11 (eotaxin-1), CCL17 (TARC), and CCL22 (MDC) and T_H1 immunity-associated IFN-γ and the chemokines CXCL9 (Mig) and CXCL10 (IP-10), as well as T regulatory-associated IL-10, were quantified. Ultrasensitive absorbance or chemiluminescense ELISAs developed in our laboratory and described in detail in previous publications[25-27] were used. World Health Organization standards were used where available. In all

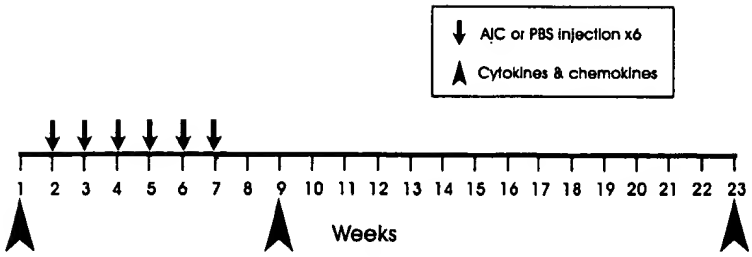

FIG 1. Time course of the study: six injections of AIC in incremental doses from 0.06 to 12 μg per injection or of placebo (PBS) were given at weekly intervals during the winter. Before the first injection and at 2 and 16 weeks after the sixth injection, ragweed-specific immunoregulatory recall cytokine and chemokine responses were evaluated in antigen-driven, short-term primary culture.

participants at all sample times, immunologic tests were performed in at least 2 independent assays, with the concentration calculated from a minimum of 3 points falling on the linear portion of sample titration curves calibrated against standards run on each plate. SEs ranged from 3% to 10%.

Statistical analysis

Data analysis included all participants, regardless of the maximum AIC dose injected during the study. Geometric means for the absolute levels of given cytokines and logs of ratios (ie, pairwise comparisons of IFN-γ/IL-5 production) are presented. *P* values are derived from a priori contrasts from repeated-measures ANOVA on logarithmically transformed data comparing AIC and placebo groups by using SAS V8.2. Significance levels are 1-tailed for cytokine and chemokine responses elicited by the sensitizing allergen (on the basis of the literature and our hypothesis that AIC administration would lead to increased T_H1 and decreased T_H2 responses) and 2-tailed for all other comparisons.

Safety

At each visit, diaries of symptoms and events were reviewed. Laboratory tests for safety, performed before the first, third, and sixth AIC injections and 2 weeks after the last injection, included complete blood count, platelet count, erythrocyte sedimentation rate, serum electrolytes, creatinine, blood urea nitrogen, glucose, aspartate transaminase, alanine transaminase, bilirubin, alkaline phosphatase, serum and total iron-binding capacity, C3, C4, and urinalysis. In addition, immunoassays for anti-nuclear antibody, anti-single-stranded DNA, and anti-double-stranded DNA were performed before the first and third study injections and repeated 4 and 16 weeks after the last injection.

RESULTS

The AIC and placebo groups were demographically and clinically similar (AIC [n = 9]: 4 men, 41 ± 13 y, 76 ± 20 kg; placebo [n = 10]: 6 men, 41 ± 13 y, 79 ± 18 kg). They did not differ significantly in the intensity of cytokine and chemokine production or in the balance between their T_H1 and T_H2 immunity at preinjection baseline ($P > .05$); however, when ragweed-specific responses were evaluated 2 and 16 weeks after the sixth and last injection, the individuals in the AIC group exhibited striking reorientation of their ragweed-specific recall responses (Table I and Fig 2).

Ragweed-specific T_H2-associated responses are reduced after injection of ISS linked to Amb a 1

Under conditions of both optimal and suboptimal allergen concentrations (100 and 10 μg/mL, respectively, with the latter used for enhanced sensitivity to possible changes in allergen-specific responses), ragweed-dependent production of the T_H2 chemokine CCL17 was markedly lower (medians, 4230 vs 1808 pg/mL, $P = .005$) in the AIC group than in the placebo group (Table I). The median intensity of these reductions was similar at 2 and 16 weeks after treatment, ranging from 2- to 4-fold under the various conditions assessed; indeed, CCL17 levels were suppressed to background levels 16 weeks after AIC injections ($P = .004$). Similarly, ragweed-dependent CCL22 responses, also initially indistinguishable in the AIC and placebo groups ($P = .14$), were reduced after AIC injections but not after placebo injections (medians of 22,925 vs 7746 pg/mL [$P < .0002$] at 2 weeks and medians of 16,317 vs 8103 [$P < .007$] at 16 weeks).

In addition, 200% to 400% dampening of some T_H2 immunity-associated cytokine responses was found. Under conditions of maximal recall responses (ragweed, 100 μg), median IL-5 recall responses were reduced by one third (medians, 293 pg/mL in the placebo group vs 198 pg/mL in the AIC group, $P < .05$) 2 weeks after the last injection, with somewhat stronger reductions evident 16 weeks after the last injection (351 vs 148 pg/mL, $P = .001$). Baseline IL-5 recall responses were indistinguishable between the groups ($P > .05$). The same picture emerged by using threshold concentrations (10 μg of ragweed: 247 vs 100 pg/mL, $P = .005$ at 16 weeks). Ragweed-driven IL-13 responses were not detectably altered before treatment ($P = .24$ and $P = .48$ for 10 and 100 μg of ragweed, respectively) at 2 weeks ($P = .46$ and $P = .48$, respectively) or 16 weeks after the last injections ($P = .12$ and $P = .27$, respectively). IL-4, CCL1, CCL2,

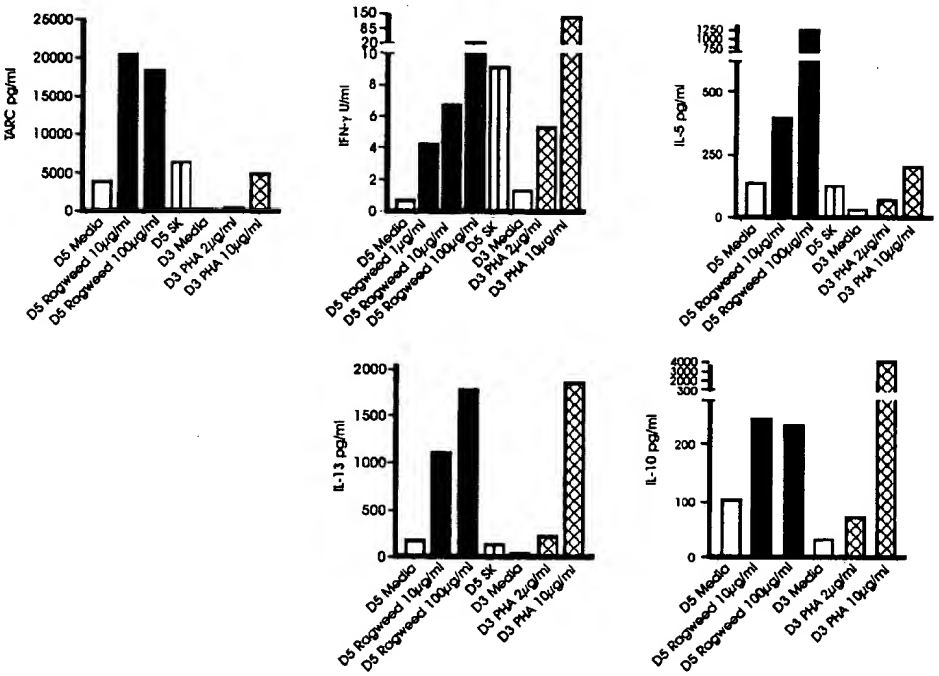

FIG 2. Expression of cytokine responses by fresh PBMCs from an individual with ragweed allergy representative of baseline results. Geometric mean responses from 5×10^5 PBMCs after 3 to 5 days of stimulation with sensitizing allergen at suboptimal (1 and 10 μg/mL) and optimal (100 μg/mL) conditions, streptokinase (5000 U/mL), and polyclonal activator PHA (suboptimal, 2 μg/mL; optimal, 10 μg/mL) and responses in unstimulated control wells were determined in tissue culture supernatants.

TABLE I. Ragweed-specific recall responses

	2 Wk after injection (pg/mL), PBS vs AIC	AIC effect	16 Wk after injection (pg/mL), PBS vs AIC	AIC effect
T_H1				
CXCL10	10,715 vs 11,561, $P > .05$	~	11,142 vs 27,925, $P < .004$	↑
CXCL9	2344 vs 4915, $P < .02$	↑	1998 vs 3751, $P > .05$	~
IFN-γ	161 vs 483, $P = .004$ to $P = .015$	↑	219 vs 207, $P > .05$	~
T_H2				
CCL17	4230 vs 1808, $P = .005$	↓	6836 vs 2697, $P = .004$	↓
CCL22	22,925 vs 7746, $P < .0002$	↓	16,317 vs 8103, $P < .007$	↓
IL-5	293 vs 198, $P < .05$	↓	351 vs 148, $P = .001$	↓
IL-13	226 vs 265, $P = .48$	~	120 vs 122, $P = .27$	~
T regulatory				
IL-10	179 vs 473, $P = .03$	↑	403 vs 590, $P > .05$	~

AIC versus placebo: ↑, significant increase; ↓, significant decrease; ~, no significant effect.

and CCL11 responses were less than the levels of detection in virtually all allergen-driven responses examined (1, 10, and 100 μg/mL ragweed).

Ragweed-specific T_H1-associated responses are increased after injection of ISS linked to Amb a 1

CXCL10 levels were similar in the treatment groups at study entry, but by 16 weeks after the last injection, as compared with the placebo group, there were 3-fold increases in the AIC group (medians, 11,142 vs 27,925 pg/mL; $P < .004$; Table I). CXCL9 responses, usually coregulated with CXCL10 and also highly IFN-γ dependent, were substantial in both treatment groups and also trended higher after AIC. Classical markers of T_H1 immunity, such as IFN-γ, were enhanced on ragweed restimulation, with individuals in the AIC group exhibiting median responses 2- or 3-fold higher than those in the placebo group (161 vs 483 pg/mL, $P = .004$ to $P = .015$) at 2 weeks after treatment. Interestingly, unlike the changes in T_H2 immunoregulatory cytokines and chemokines, these increases in IFN-γ did not persist at 16 weeks (219 vs 207 pg/mL, $P = .24$).

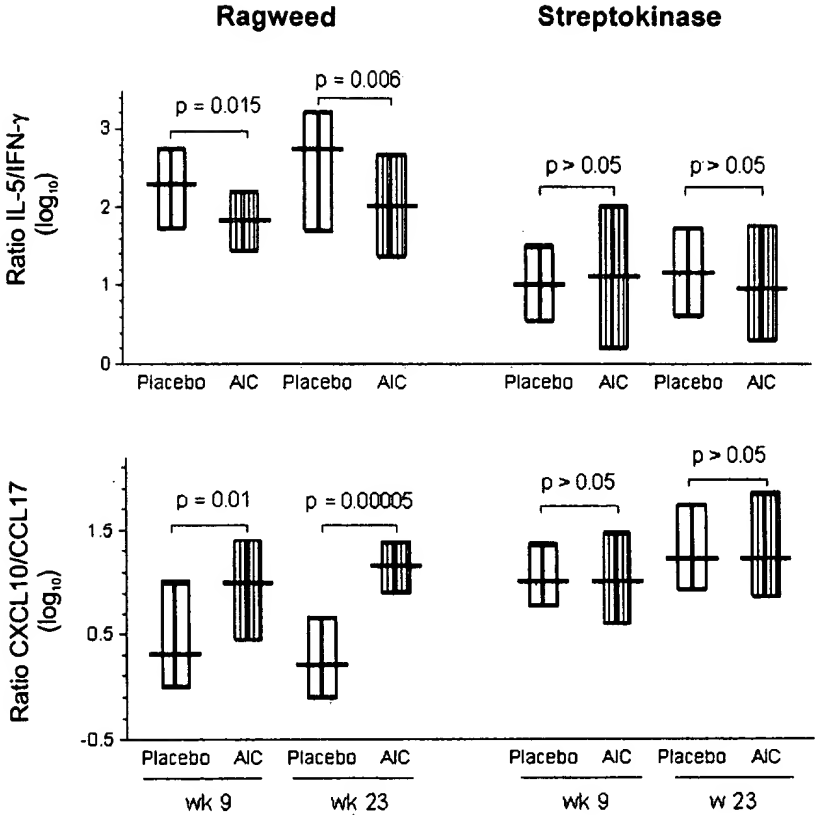

FIG 3. Modification of ragweed-driven, but not streptokinase-driven, cytokine and chemokine responses in individuals with ragweed allergy after AIC administration. Results are displayed as the median log ratio of IL-5/IFN-γ production and CXCL10/CCL17 production. *Boxes* indicate the limits of the 25th and 75th percentiles (AIC group, n = 9; placebo [PBS] group, n = 10) for each comparison. *P* values represent statistical significance from repeated-measures ANOVA on logarithmically transformed data.

IL-10 responses, of interest because of their potential role in regulatory T-cell biology, exhibited a transient increase in the AIC group compared with the placebo group at 2 weeks (473 vs 179 pg/mL, $P = .03$) that was not evident at 16 weeks.

Shifting the immunologic balance away from T_H2-dominated immunity

The ratios of cytokine and chemokine responses were examined to obtain a broader measure of the effect of AIC administration on the balance of immunity than is possible through examination of individual cytokines. Median ratios of ragweed allergen-driven T_H2/T_H1 (IL-5/IFN-γ) recall responses (Fig 3) demonstrate striking reductions. Whereas responses to sensitizing allergen were indistinguishable in the 2 groups before AIC or placebo injection ($P = .22$), AIC led to 3- to 10-fold changes in the balance of the ragweed-specific recall response, shown as ragweed-stimulated IL-5/IFN-γ. This redirection continued to the end of the study (median ratios of 200:1 vs 69:1 and 562:1 vs 64:1 at 2 and 16 weeks, respectively, after the last injection). Use of threshold antigen stimulation conditions (ragweed, 10 μg/mL) also demonstrated placebo and AIC groups to be indistinguishable initially ($P = .24$), followed by redirection of the T_H2/T_H1 response at 2 and 16 weeks (257 vs 100, $P = .025$) after treatment.

The ratios of T_H1/T_H2 chemokine responses were indistinguishable between the groups before the injections (all $P > .05$). The AIC group exhibited a 5-fold shift in the ragweed-specific balance 2 weeks after the last injection ($P = .01$) and a 9-fold shift 16 weeks after the last injection (median CXCL10/CCL17 ratios of 1.6 vs 14.5 in the placebo group vs the AIC group, respectively; $P < .00005$; Fig 3). Comparison of a range of other T_H1/T_H2 indicators revealed similar 2- to 9-fold shifts in allergen-driven recall responses (IFN-γ/IL-5, $P < .05$, and CXCL10/CCL17, $P < .01$ to $P < .0001$).

Global effect of AIC administration

In contrast to the effect of AIC on ragweed-specific recall responses, neither T_H1 nor T_H2 recall responses to streptokinase (Fig 3 and Table II) nor the global capacity to respond after polyclonal activation with PHA were generally altered. In streptokinase-driven recall responses, IFN-γ ($P = .75$ to $P = .97$) and CXCL10 ($P = .39$ to $P = .99$), as well as CCL17 ($P = .51$ to $P = .93$), and IL-5

(P = .21 to P = .44), were unaffected when comparing the AIC group and the placebo group before the injections and at 2 and 16 weeks after the last injection.

Mirroring cytokine responses, the ratios of CXCL10/CCL17 production in unstimulated medium controls (P = .38 to P = .78), in streptokinase-stimulated cultures (P = .99 to P = .94; Fig 3), or in cultures stimulated with PHA under threshold (P = .59 to P = .64) or maximal (P = .94 to P = .65) activation conditions did not differ detectably between the placebo and AIC groups.

Adverse effects

All 19 participants completed the series of injections and all related tests. Five of the 9 participants receiving AIC did not require any dose adjustments and tolerated the sixth and final dose of 12 μg (total dose, 28 μg), whereas 4 of the 9 participants required a reduced AIC dose as per protocol because of local reactions involving some combination of itch, pain, warmth, and redness. Nine of the 10 participants receiving placebo did not require a dose reduction. No clinically significant systemic or local allergic reactions were associated with AIC or placebo injections. No individual withdrew from the study because of adverse effects. Reported adverse events were generally mild and considered to be unrelated to AIC or placebo injections. No clinically relevant changes in hematology or blood chemistry test results were found.

DISCUSSION

In this proof-of-concept study of a novel approach to immune modulation in humans, we determined the effect of AIC on ongoing immunoregulatory responses and investigated its safety. We demonstrated for the first time that human systemic in vivo T_H2 cytokine and chemokine ragweed-specific recall responses and IL-5, CCL17, and CCL22 levels readily detectable 6 months after the end of the ragweed season were markedly reduced after AIC, but not placebo, injections. In some individuals, reductions to background levels occurred. Interestingly, IL-13 recall responses to ragweed were largely unaffected under the conditions tested, and IL-4 responses to ragweed were generally less than detection limits. In contrast, ragweed-specific T_H1 immune responses included transiently increased IFN-γ and CXCL9 production and long-term increases in CXCL10 production in the AIC group compared with the placebo group. These allergen-dependent responses are strictly dependent on $CD4^+$ T-cell activation, MHC class II antigen presentation, and costimulation through CD80/CD86 pathways (Stinson MJ, et al, manuscript in preparation). Given the small number of individuals in the study, no post hoc analysis of the relationship between maximum total dose of AIC injected and immunologic responses was attempted. Although only 5 individuals received the full dose of AIC, data from all individuals in the AIC group, regardless of the total AIC dose injected, were compared with data from all individuals in the placebo group.

Different ISSs exhibit different levels of activity in different species.[17,28-31] In some murine studies, ISS-driven changes have been global rather than allergen specific, a potential concern in human immunotherapy. In marked contrast, in this study immunologic changes were mainly restricted to ragweed-specific responses, possibly because the amount of ISS administered was substantially less than that in most animal studies to date. Although mice are typically given 2 to 5 injections of 50 to 100 μg (approximately 10-15 mg/kg total) of ISSs mixed with antigen, participants in this study who received the full course of AIC injections received approximately 0.3 μg/kg ISS linked to Amb a 1. Selection of this lower dose was based on animal studies demonstrating that linkage of antigen to ISS markedly enhances its immunogenicity and reduces its allergenicity in comparison with simply mixing it with an ISS.[20,28,32,33] Therefore the 2- to 5-fold increases in T_H1 cytokine and chemokine profiles and similar reductions in T_H2 cytokines (predominately IL-5) seen in successful murine studies modifying established T_H2-biased immunity[17,18,29] were achieved in this human study by using 3000-fold less ISS linked to antigen (comparable with 30,000- to 50,000-fold less ISS mixed with antigen). Clearly, only very small amounts of linked ISSs are required to alter the immunoregulatory cytokine and chemokine recall response to sensitizing allergen in humans, and under these conditions, the immune redirection achieved does not result in a potentially dangerous global redirection of immune capacity but rather is restricted to the specific immune responses targeted.

Another important distinction between murine studies and the human study reported here concerns the nature of the experimental protocol and duration of biologic effects induced. Most studies of ISS administration aiming to downregulate ongoing T_H2 responses in animals have involved injections immediately (usually 1-3 days) before evaluation of recall cytokine responses,[17,18,29] irrespective of whether ISSs were given without allergen,[16,34] with allergen added, or with allergen chemically linked to the ISS. However, in contrast to ISSs used to influence initial responses in naive animals in which induction of T_H1-biased immunity persists for more than 1 year, activity of ISSs in ongoing murine allergy models has generally been found to be short lived, persisting between 1 and 2 months, after which time the specific recall responses in animals receiving ISSs are often indistinguishable from the responses in those receiving placebo.[17] We therefore designed this study to evaluate potential short-term (2 weeks after completion of treatment) and sustained (16 weeks later) alterations in responses to ragweed and unrelated antigen. Several observations are apparent. First, changes in allergen-dependent recall responses, the expression of which is associated with T_H1 (CXCL9, CXCL10) and T_H2 (CCL17, CCL22) responses, were readily observed indicators of T_H1/T_H2 immunity. Second, unlike in murine studies, ISS administration in humans resulted in sustained alterations in the T_H1/T_H2 balance of recall responses, which lasted for at least 16

TABLE II. Responses to streptokinase and PHA

	2 Wk after injection (pg/mL), PBS vs AIC	AIC effect	16 Wk after injection (pg/mL), PBS vs AIC	AIC effect
Streptokinase				
T_H1				
CXCL10	27,227 vs 21,380, $P = .39$	~	33,113 vs 32,359, $P = .99$	~
IFN-γ	736 vs 724, $P = .97$	~	518 vs 449, $P = .75$	~
T_H2				
CCL17	2617 vs 2059, $P = .51$	~	1920 vs 1984, $P = .93$	~
IL-5	63 vs 81, $P = .44$	~	64 vs 40, $P = .21$	~
IL-13	130 vs 210, $P = .02$	↓	50 vs 77, $P = .05$	~
T regulatory				
IL-10	246 vs 328, $P = .69$	~	307 vs 357, $P = .53$	~
PHA				
T_H1				
CXCL10	7244 vs 7079, $P = .98$	~	15,488 vs 12,882, $P = .57$	~
IFN-γ	3738 vs 2553, $P = .53$	~	6325 vs 3347, $P = .28$	~
T_H2				
CCL17	2208 vs 1959, $P = .71$	~	4064 vs 3415, $P = .57$	~
IL-5	147 vs 145, $P = .97$	~	103 vs 128, $P = .34$	~
IL-13	556 vs 706, $P = .38$	~	399 vs 424, $P = .79$	~
T regulatory				
IL-10	5653 vs 3678, $P < .05$	↓	5767 vs 3328, $P < .02$	↓

AIC versus placebo: ~, no significant effect; ↓, decrease.

weeks. Interestingly, although enhanced T_H1 cytokine and chemokine synthesis and reduced T_H2 cytokine and chemokine synthesis were both clearly evident in the ragweed-specific response 2 weeks after AIC treatment, these changes were relatively transient. The most durable effect of AIC injections, seen 16 weeks later, was the reduced T_H2 regulatory responses.

This may have important implications for immunomodulation in humans. Although immediate hypersensitivity diseases are classically defined as T_H2 dependent, there is considerable evidence of enhanced IFN-γ synthesis during chronic allergen stimulation.[1] Excessive T_H1 cytokine and chemokine production could potentially have negative consequences by exacerbating, rather than reducing, this inflammatory process. Our observations in this translational research suggest that ISS administration by means of an allergen-linked molecule may lead to pronounced inhibition of allergen-specific T_H2 responses without chronically high levels of IFN-γ expression. ISSs are markedly lower in toxicity than other danger signals such as LPSs and other pathogen-associated molecular patterns to which the innate immune system is programmed to respond promptly and vigorously.

AIC appeared to have a good safety profile, as indicated by 100- to 300-fold decreased allergenicity[23] and few adverse effects, despite the high doses of ragweed allergen injected in this study relative to those injected in conventional allergen-specific immunotherapy regimens. History, physical examination, and clinical laboratory evaluations did not suggest any evidence of global immune stimulation. The local reactions observed with AIC injections were similar to those observed with licensed ragweed allergen extracts and were consistent with pre-existing ragweed allergy.

In summary, we have demonstrated for the first time that injections of AIC in humans with ragweed allergy resulted in a marked shift in systemic in vivo allergen-specific immunoregulatory responses characterized by transiently enhanced IFN-γ and longer-term inhibition of T_H2 cytokine and chemokine responses, including IL-5, CCL17, and CCL22. The net effect of this intervention was a shift in the T_H1/T_H2 immunoregulatory cytokine and chemokine balance of the ragweed-specific response without global effects on the immune system because the effects of AIC administration were restricted to the sensitizing allergen, with minimal detectable differences in responses to an unrelated antigen or to broader immune activation.

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REFERENCES

1. Holgate ST. The epidemic of allergy and asthma. Nature 1999; 402(suppl):B2-4.
2. Umetsu DT, Akbari O, Dekruyff RH. Regulatory T cells control the development of allergic disease and asthma. J Allergy Clin Immunol 2003;112:480-7.
3. Durham SR, Till SJ. Immunologic changes associated with allergen immunotherapy. J Allergy Clin Immunol 1998;102:157-64.
4. Secrist H, Chelen CJ, Wen Y, Marshall JD, Umetsu DT. Allergen immunotherapy decreases interleukin 4 production in CD4+ T cells from allergic individuals. J Exp Med 1993;178:2123-30.
5. Jutel M, Pichler WJ, Skrbic D, Urwyler A, Dahinden C, Muller UR. Bee venom immunotherapy results in decrease of IL-4 and IL-5 and increase of IFN-gamma secretion in specific allergen-stimulated T cell cultures. J Immunol 1995;154:4187-94.

6. Jutel M, Akdis M, Budak F, Aebischer-Casaulta C, Wrzyszcz M, Blaser K, et al. IL-10 and TGF-beta cooperate in the regulatory T cell response to mucosal allergens in normal immunity and specific immunotherapy. Eur J Immunol 2003;33:1205-14.
7. Nelson HS. Immunotherapy for inhalant allergens. In: Adkinson NF Jr, Yunginger JW, Busse WW, Bochner BS, Holgate ST, Simons FER, editors. Middleton's allergy: principles and practice. 6th ed. St Louis: Mosby, Inc; 2003. p. 1455-73.
8. Bousquet J, Lockey R, Malling HJ. Allergen immunotherapy: therapeutic vaccines for allergic diseases. A WHO position paper. J Allergy Clin Immunol 1998;102:558-62.
9. Krieg AM, Yi AK, Matson S, Waldschmidt TJ, Bishop GA, Teasdale R, et al. CpG motifs in bacterial DNA trigger direct B-cell activation. Nature 1995;374:546-9.
10. Hsu CH, Chua KY, Tao MH, Lai YL, Wu HD, Huang SK, et al. Immunoprophylaxis of allergen-induced immunoglobulin E synthesis and airway hyperresponsiveness in vivo by genetic immunization. Nat Med 1996;2:540-4.
11. Roman M, Martin-Orozco E, Goodman JS, Nguyen MD, Sato Y, Ronaghy A, et al. Immunostimulatory DNA sequences function as T helper-1-promoting adjuvants. Nat Med 1997;3:849-54.
12. Cohen AD, Boyer JD, Weiner DB. Modulating the immune response to genetic immunization. FASEB J 1998;12:1611-26.
13. Roy K, Mao HQ, Huang SK, Leong KW. Oral gene delivery with chitosan—DNA nanoparticles generates immunologic protection in a murine model of peanut allergy. Nat Med 1999;5:387-91.
14. Kovarik J, Bozzotti P, Love-Homan L, Pihlgren M, Davis HL, Lambert PH, et al. CpG oligodeoxynucleotides can circumvent the Th2 polarization of neonatal responses to vaccines but may fail to fully redirect Th2 responses established by neonatal priming. J Immunol 1999;162: 1611-7.
15. Peng Z, Wang H, Mao X, HayGlass KT, Simons FER. CpG oligodeoxynucleotide vaccination suppresses IgE induction but may fail to downregulate ongoing IgE responses in mice. Int Immunol 2001;13:3-11.
16. Sur S, Wild JS, Choudhury BK, Sur N, Alam R, Klinman DM. Long term prevention of allergic lung inflammation in a mouse model of asthma by CpG oligodeoxynucleotides. J Immunol 1999;162: 6284-93.
17. Horner AA, Van Uden JH, Zubeldia JM, Broide D, Raz E. DNA-based immunotherapeutics for the treatment of allergic disease. Immunol Rev 2001;179:102-18.
18. Kline JN. Effects of CpG DNA on Th1/Th2 balance in asthma. Curr Top Microbiol Immunol 2000;247:211-25.
19. Klinman DM, Barnhart KM, Conover J. CpG motifs as immune adjuvants. Vaccine 1999;17:19-25.
20. Tighe H, Takabayashi K, Schwartz D, Van Nest G, Tuck S, Eiden JJ, et al. Conjugation of immunostimulatory DNA to the short ragweed allergen Amb a 1 enhances its immunogenicity and reduces its allergenicity. J Allergy Clin Immunol 2000;106:124-34.
21. Bohle B, Jahn-Schmid B, Maurer D, Kraft D, Ebner C. Oligodeoxynucleotides containing CpG motifs induce IL-12, IL-18 and IFN-gamma production in cells from allergic individuals and inhibit IgE synthesis in vitro. Eur J Immunol 1999;29:2344-53.
22. Hartmann G, Krieg AM. CpG DNA and LPS induce distinct patterns of activation in human monocytes. Gene Ther 1999;6:893-903.
23. Marshall JD, Abtahi S, Eiden JJ, Tuck S, Milley R, Haycock F, et al. Immunostimulatory sequence DNA linked to the Amb a 1 allergen promotes T(H)1 cytokine expression while downregulating T(H)2 cytokine expression in PBMCs from human patients with ragweed allergy. J Allergy Clin Immunol 2001;108:191-7.
24. Marshall JD, Fearon K, Abbate C, Subramanian S, Yee P, Gregorio J, et al. Identification of a novel CpG DNA class and motif that optimally stimulate B cell and plasmacytoid dendritic cell functions. J Leukoc Biol 2003;73:781-92.
25. Li Y, Simons FER, HayGlass KT. Environmental antigen-induced IL-13 responses are elevated among subjects with allergic rhinitis, are independent of IL-4, and are inhibited by endogenous IFN-gamma synthesis. J Immunol 1998;161:7007-14.
26. Gangur V, Simons FER, HayGlass KT. Human IP-10 selectively promotes dominance of polyclonally activated and environmental antigen-driven IFN-gamma over IL-4 responses. FASEB J 1998;12: 705-13.
27. Lewkowich IP, Campbell JD, HayGlass KT. Comparison of chemiluminescent assays and colorimetric ELISAs for quantification of murine IL-12, human IL-4 and murine IL-4: chemiluminescent substrates provide markedly enhanced sensitivity. J Immunol Methods 2001;247: 111-8.
28. Santeliz JV, Van Nest G, Traquina P, Larsen E, Wills-Karp M. Amb a 1-linked CpG oligodeoxynucleotides reverse established airway hyperresponsiveness in a murine model of asthma. J Allergy Clin Immunol 2002;109:455-62.
29. Krieg AM. The role of CpG motifs in innate immunity. Curr Opin Immunol 2000;12:35-43.
30. Gurunathan S, Klinman DM, Seder RA. DNA vaccines: immunology, application, and optimization. Annu Rev Immunol 2000;18:927-74.
31. Bohle B. CpG motifs as possible adjuvants for the treatment of allergic diseases. Int Arch Allergy Immunol 2002;129:198-203.
32. Cho HJ, Takabayashi K, Cheng PM, Nguyen MD, Corr M, Tuck S, et al. Immunostimulatory DNA-based vaccines induce cytotoxic lymphocyte activity by a T-helper cell-independent mechanism. Nat Biotechnol 2000;18:509-14.
33. Shirota H, Sano K, Kikuchi T, Tamura G, Shirato K. Regulation of T-helper type 2 cell and airway eosinophilia by transmucosal coadministration of antigen and oligodeoxynucleotides containing CpG motifs. Am J Respir Cell Mol Biol 2000;22:176-82.
34. Magone MT, Chan CC, Beck L, Whitcup SM, Raz E. Systemic or mucosal administration of immunostimulatory DNA inhibits early and late phases of murine allergic conjunctivitis. Eur J Immunol 2000;30: 1841-50.

remission was obtained in 31 cases (82%), partial response in 2 cases (5%), progression in 2 (5%), and 3 patients were not evaluated. Nineteen patients relapsed (50%), ten (26%) with systemic relapse, four (13%) local and five (13%) locoregional failure. At the moment of the analysis the median follow-up is 64 months (limits 12-233), 14 patients are alive and disease-free (37%), 5 patients are alive in relapse (13%) and 19 patients died, 16 (42%) secondary to tumor progression and 3 (8%) by other causes. Median survival of 54 months and ten year survival of 38%. Median survival in patients treated before 1990 was 47 months; in patients treated after that date the median survival has not been arised (p=0.49). Median survival in patients treated only with RT was of 47 months and in patients in which CT was added the survival was of 53 months (p=0.19). CONCLUSIONS: Undifferentiated nasopharyngeal carcinoma is an uncommon tumor. Median age of the patients is lower than age of diagnosis of head and neck cancer. The disease appears at advanced stages. RT is the main therapeutic modality and the overall response rate must be near 70%. Combination treatment (RT-CT) improves the results and is considered today the standard treatment.

MELANOMA AND SARCOMA

5770 Incidence, prevalence, phenotype and biologic spectrum of gastrointestinal stromal cell tumors (GIST) - A population-based study of 600 cases

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The recent refinement of diagnostic criteria for GIST and particularly the ability to detect the KIT receptor immunohistochemically with CD117 have now made it possible to determine the true incidence and prevalence of GIST. With the development of the remarkably effective new treatment modality Glivec (imatinib, formerly STI571, Novartis), a KIT receptor tyrosine kinase inhibitor, further definition of diagnostic criteria and prognostic markers has become extremely important. Most previous large studies of GIST are probably biased regarding its spectrum of biologic behavior, since they have been based primarily on cases that have been diagnosed and/or treated at a referral center. Our study aims to analyze a well-defined population from southwestern Sweden (population approximately 1.5 million) where all histopathologic diagnoses have been made in four pathology laboratories. All potential cases of GIST diagnosed between 1983 and 2000 have been retrieved and reviewed, resulting in a total of 600 GIST fulfilling diagnostic criteria which included CD117 immunoreactivity. Histologic and immunophenotypic features have been analyzed as well as details of clinical presentation, behavior, treatment, and prognosis. In addition, material has been obtained for a tissue array bank and for KIT mutation analysis. All data is currently being subjected to multivariate statistical analysis. Our results indicate that: 1) symptomatic/clinically detected GIST occur with an incidence of approximately 20 cases/million inhabitants per year; 2) GIST is a rather common incidental finding at surgery, endoscopy, or autopsy; 3) histologically GIST has been underrecognized and its malignant potential has been underestimated; 4) the histologic spectrum of GIST is wider than previously recognized; and 5) a small subgroup of GIST lacking CD117 immunoreactivity can be defined.

5780 Fotemustine (F) *versus* Dacarbazine (DTIC) as first line treatment in disseminated malignant melanoma (MM) with or without brain metastases (BM): A randomized phase III trial

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This phase III study compared F (100mg/m² iv weekly, 3 weeks) and DTIC (250 mg/m²/d iv, 5 days every 3 weeks, 2 cycles) in terms of objective response rate (OR), safety profile, quality of life (QoL) using QLQ-C30 scores, and overall survival (OS). Non-progressive patients (pts) received a maintenance therapy every 3 weeks (F 100 mg/m² or DTIC 250 mg/m²/dx5). From 02.98 to 09.00, 229 pts were randomized to receive F (n=112) or DTIC (n=117). In the Full Analysis Set, the OR was 15.5% (F) *versus* 7.2% (DTIC) (p=0.053). In pts without BM at inclusion (n=182), the median time of BM occurence was 22.7 (F) *vs* 7.2 months (DTIC) (p=0.059). The main toxicity was grade 3-4 neutropenia (51% of pts in F arm *vs* 5% in DTIC arm) and thrombocytopenia (43% in F arm *vs* 6% in DTIC arm) occuring mainly during induction: Severe non-hematological toxicity was infrequent, including pain (4 pts in F *vs* 17 in DTIC) and nausea-vomiting (3 pts in F *vs* 4 in DTIC). No toxic death occured. No significant difference was noted for QoL results between arms (n=156). Subgroup analyses showed a general trend for deteriorating QoL in progressive pts and stable or improving QoL for others. At time of analysis, the median OS was 7.4 (F) *vs* 5.8 months (DTIC) (p=0.073). A positive trend in favor of F in terms of OR, OS, and time to BM occurence was seen in first line treatment of MM. Both treatments had acceptable safety profiles and no difference was noted in QoL scores.

5790 Phase I/II study with CpG 7909 as adjuvant to vaccination with MAGE-3 protein in patients with MAGE-3 positive tumors

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Vaccination is a new rapidly expanding modality in antitumor immunotherapy. To improve effectiveness of anticancer vaccines powerful immunological adjuvants are needed. Recently, specific synthetic oligodeoxynucleotids sequences demonstrated immunostimulatory effects. The dinucleotide CpG 7909 is a novel promising adjuvant for induction of both humoral and cell-mediated immune responses. Furthermore, MAGE-3 encoded tumor-specific antigens are established targets for anticancer vaccine therapy. In a ongoing phase I/II study patients with metastatic MAGE-3 positive melanoma were vaccinated with CpG 7909 and MAGE-3 protein to evaluate safety, tolerability, immunological reactiveness and antitumor activity. Eligibility was according to standard criteria. Patients known with autoimmune diseases were excluded. Patients were vaccinated intramuscularly on days 1, 22, 43 and 64. Patients without progression received further vaccinations on days 92, 113 and 134. We studied 2 different dose levels of CpG 7909 (500 and 1000 microgram). The MAGE-3 protein dose was fixed at 300 microgram. Presently 13 patients have been enrolled, 8 in cohort 1 and 5 in cohort 2. Eleven patients received at least 2 vaccinations, one 2, one 3, two 4, one 6, four 7, one 12 and one 15 vaccinations, respectively. Two patients in cohort 1 were not evaluable due to rapid progressive disease (1) and intercurrent neurologic paraneoplastic syndrome (1). Treatment was well tolerated with transient grade I and II toxicities consisting of fever, fatigue, myalgia, headache, nausea/vomiting, tenderness and skin reaction at the injection site with no apparent CpG 7909 dose-related differences. One patient showed stable disease (12+ months) and 1 patient developed a partial response (9+ months). Preliminary immunological analysis showed an increase (10-150X) in anti-MAGE-3 antibody titers with a

stronger reaction in the 1000 microgram CpG dose cohort. In conclusion, vaccination with the immunological adjuvant CpG 7909 up to a dose of 1000 microgram in combination with the MAGE-3 protein is well tolerated and safe. The preliminary results of this study support further research.

580O Phase I trial of dexosome vaccine for patients with advanced melanoma: Final results

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Dexosomes (Dendritic cell-derived EXosomes), 60-90nm vesicles released by immature monocyte-derived dendritic cells (MD-DC), can be efficiently loaded with tumor peptides to induce potent T cell stimulation. A Phase I safety and feasibility study of DEX vaccine therapy was conducted in 15 patients with advanced melanoma. Eligibility requirements: Stage III/IV melanoma, HLA A1/B35 and DP04 haplotype, tumor Mage-3 expression and + recall skin testing. The DEX were isolated from DC culture supernatant from a single leukapheresis (except in 2 pt). Tumor peptides (Mage-3.A1/Mage-3.DP04 were loaded onto the DC during culture (6 pts); or loaded directly onto the purified dexosomes (9 pts). Escalating doses of cryopreserved DEX were administered by 4 weekly id/sc injections, then every 3 weeks in patients who achieved stable disease or a tumor regression.. Fifteen patients have entered the study and 13 have completed vaccine therapy as of June 1st, 2002. Patient characteristics: median age 59 (range 29-76); M:F, 8:7; Stage; III 5 pts, IV 10 pts; 53% had prior chemotherapy; 27% had prior immunotherapy; 4 patients had an elevated LDH at entry. All patients had progressive disease before treatment, sites of metastatic disease included skin, lung, nodes and liver. Results: The vaccine therapy was well tolerated, without evidence of serious toxicity. Among the 13 patients who have completed treatment, one patient with Stage III disease achieved stable disease following the first 4 injections, received 8 additional vaccine injections with continued stable disease 15+ months. One partial response on lymph nodes and one "mixed" response have been observed at the highest level. Immunomonitoring results will be presented including lymphocytes microcultures screened by MAGE.3-A1/B35 tetramers allowing isolation of specific CTL clones in stable or responding patients. This phase I trial demonstrate that administration of DEX is feasible and safe with encouraging clinical and immunological responses to support a phase II study in melanoma. Clinical and immune responses of all 15 patients will be presented and updated.

581P Prognostic factors in radically resected malignant melanoma

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Introduction: The c-kit gene encodes a transmembrane tyrosine kinase receptor and plays an important role in tumor growth, invasion and metastatic spread of malignant melanoma. Expression of c-kit was investigated in preclinical studies. No large clinical study has been done thus far. Patients and Methods: Primary tumor specimens of 189 radically resected patients with stage I and II malignant melanoma were examined for the presence of c-kit expression. Formalin-fixed, paraffin embedded, tissues were stained with the polyclonal rabbit anti-human c-kit antibody (Dako®). The univariate and multivariate analysis of c-kit expression and other clinical (disease free survival) or pathological features (Breslow, Clark, microscopic ulceration) have been performed. Results: In the population of 189 early stages melanoma patients occurred 37 recurrences. Among investigated samples were 121 (64%) negative for c-kit expression, 57 (30%) samples were positive in less than 50% of cells and 11 (6%) samples were positive in more than 50% of cells. In univariate analyses, high Breslow, Clark and the presence of microscopic ulceration were significantly associated with shorter disease free survival (respectively: $p<0.000001$; $p<0.0007$; $p<0.0001$). c-kit overexpression almost reached statistical significance ($p<0.0594$) as a negative prognostic factor. In multivariate analyses the combination of Breslow, ulceration and c-kit appeared useful in the assessment of patient prognosis (respectively: $p<0.0002$, $p<0.0062$, $p<0.0142$). When all parameters were correlated to each other the only strong positive correlation was found between Breslow and Clark ($r=0,61$; $p = 0.0001$). Conclusion: Breslow, Clark and microscopic ulceration were only statistically significant prognostic factors. However, c-kit expression almost reached the significance. The combination of Breslow, ulceration and c-kit assessment appeared to be ideal combination in determination of prognosis. The small number of events in the study group might influence these results. Acknowledgment: This research was supported by the grants of the League Against Cancer, Terry Fox Foundation and CEZ a.s.. Thanks to Dr. Julis for the c-kit expression determination.

582P Accuracy of sentinel lymph node and positron emission tomography scanning in the detection of micro-metastases of malignant melanoma

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Purpose: The pathologic status of the sentinel lymph node (SN) is a powerful indicator of prognosis in patients with melanoma. 18-fluoro-2-deoxy-D-glucose positron emission tomography (18FDG-PET) scanning is a non invasive imaging technique that can detect clinically silent metastases. The aim of this study is to compare the use of 18FDG-PET and sentinel lymph node biopsy (SNB) in detecting metastatic disease in the regional draining nodes of cutaneous malignant melanoma. Patients and methods: Twenty two consecutive patients (14 women and 8 men; mean age 48 years) with primary cutaneous melanoma = 1mm or <1mm and Clark level IV-V, ulceration or lymphovascular invasion were recruited between March 1998 and January 2002. Mean Breslow thickness was 1.9 mm (range 0.75- 7 mm). The sites of primary melanoma were 11 on the extremities, 6 on the trunk and 5 on head and neck. Ulceration was present in 2 lesions. Distant metastases were excluded by clinical exploration, biochemical profile and chest x-ray. PET scanning was followed by a preoperative lymphoscintigraphy with 99mTc sulphur colloid to identify SNs. All patients with positive SNB underwent therapeutic lymph node dissection (LND). Results: The SNs were identified in 100% of the patients. A total of 32 SNs were detected and removed. In four patients (18%), histopathology revealed lymph node metastases (4 lymph nodes). Regional LND demonstrated one more lymph node metastasis in one patient. In none of these 4 patients PET scans identified metastatic disease in the SN or draining basin. Two patients had positive PET scans suspicious of metastatic disease, both of them with a negative SNB. No patient has developed recurrent disease (mean follow-up, 7 mounths). Conclusions: This study demonstrates the limitations of 18FDG-PET in detecting lymph node micrometastases in patients with primary malignant melanoma compared with SNB.

583P Low prevalence of microsatellite instability in malignant melanoma

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Background: Malignant melanoma (MM) is thought to arise by sequential accumulation of genetic alterations in normal melanocytes. To clearly assess the role of genetic instability in MM, we have compared allelic alterations (detected as microsatellite instability; MSI) between primary tumors and synchronous or asynchronous metastases obtained from the same MM patients. Methods: Paraffin-embedded tissue microdissections from 56 MM patients were screened for MSI by PCR-based microsatellite analysis. MSI was studied at five loci containing single- or dinucleotide repeat sequences and mapping to different chromosomal locations. Tumors were classified as MSI+ when at least two markers displayed mutant alleles in tumor DNA compared to corresponding normal tissue DNA. Results: Presence of MSI, which may reflect a defect in genes involved in DNA replication fidelity, was observed in 5 (9%) out of 56 primary MM tumors. When the same series of patients has been analyzed for the presence of MSI in the corresponding metastases, we found a higher incidence (7/42, 17%) of

Induction of Systemic T_H1-Like Innate Immunity in Normal Volunteers Following Subcutaneous but Not Intravenous Administration of CPG 7909, a Synthetic B-Class CpG Oligodeoxynucleotide TLR9 Agonist

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Abstract: Subcutaneous injection of normal human volunteers with a B-class CpG oligodeoxynucleotide (ODN) TLR9 agonist, CPG 7909, induced a T_H1-like pattern of systemic innate immune activation manifested by expression of IL-6, IL-12p40, IFN-α, and IFN-inducible chemokines. Serum IP-10 was found to be the most sensitive assay for subcutaneous CPG 7909 stimulation; its level was significantly increased in all subjects at all dose levels, including the lowest tested dose of just 0.0025 mg/kg. This pattern of chemokine and cytokine induction was markedly different from that previously reported to be induced by TLR9 stimulation in rodents, most likely reflecting species-specific differences in the cell types expressing TLR9. Subcutaneous CPG 7909 injection induced transient shifts in blood neutrophils, lymphocytes, and monocytes, consistent with the increased chemokine expression. Levels of acute phase reactants such as C-reactive protein were also increased. A second subcutaneous CPG 7909 injection administered 2 weeks after the first elicited similar immune responses, showing little or no tolerance to the effects of repeated in vivo TLR9 stimulation. Subjects developed dose-dependent transient injection site reactions and flu-like symptoms but otherwise tolerated injection well, with no evidence of organ toxicity or systemic autoimmunity. The activation of innate immunity was dependent on the route of ODN administration, since intravenous injection caused no such effects. These studies indicate that in vivo activation of TLR9 by subcutaneous administration of CPG 7909 could be a well-tolerated immunotherapeutic approach for induction of T_H1 innate immune activation.

Key Words: CpG, IP-10, immune activation, dendritic cell, lymphocyte activation, B cell, Toll-like receptor

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Several generations of immunologists have recognized the potent adjuvant activity of bacterial extracts, such as complete Freund's adjuvant (CFA). Certain conserved microbe-specific molecules within bacterial extracts are detected by the innate immune system through receptors such as the Toll-like receptors (TLRs), of which 10 members have been identified in humans.[1] TLRs are expressed on selected innate immune cells such as dendritic cell (DC) subsets, macrophages, monocytes, and neutrophils and trigger cell activation in response to their ligands, such as peptidoglycans, zymosan, lipopolysaccharides, and bacterial DNA.[1,2] The immune stimulatory effects of bacterial DNA are a consequence of the presence of unmethylated CpG dinucleotides in particular base contexts, termed "CpG motifs."[3] CpG motifs are common in bacterial DNA but are underrepresented and methylated in vertebrate DNA.[4,5]

The receptor used by the innate immune system for detecting CpG motifs is TLR9,[6] which in humans is expressed in B cells and plasmacytoid dendritic cells (pDCs),[7–9] and in mice additionally in myeloid DC, and monocyte/macrophages.[7,10,11] TLR9 can be activated by synthetic oligodeoxynucleotides (ODNs) of about 8 to 30 bases in length that contain a nuclease-resistant phosphorothioate backbone and one or more CpG motifs. While most CpG motifs are effective in a wide range of species, there are species-specific differences in the optimal CpG motif, with GACGTT being optimal for mice[3,12,13] and GTCGTT for humans.[14,15]

Studies in mice indicate that the immune effects of activating TLR9-expressing cells with CpG ODNs are T_H1-like and may be considered in two stages: an early innate immune activation and a later enhancement of adaptive immune responses. Within minutes of exposure to CpG, the ODNs appear to enter an endosomal compartment where they interact with TLR9, leading to the activation of cell signaling pathways that culminate in the expression of costimulatory molecules, resistance to apoptosis, and secretion of proinflammatory cytokines and chemokines such as TNF-α, IL-6, IL-12, IFN-γ, and IFN-γ-inducible protein 10 (IP-10) or CXCL-10.[16–18] In vivo after either subcutaneous (SC) or intravenous (IV) administration of CpG ODNs to mice, peak serum levels of cytokines are typically observed at approximately 1 hour for TNF-α and 2 to 4 hours after injection for the others.[19,20] These cytokines and chemokines trigger within hours a wide range of secondary effects, such as NK cell activation.[21] This innate immune activation and DC maturation is followed by

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the generation of adaptive immune responses. B cells are strongly co-stimulated if they bind specific antigen at the same time as TLR9 stimulation.[3] This selectively enhances the development of antigen-specific antibodies, especially of the IgG2a isotype associated with T_H1-like immune responses in mice. In mouse disease models, the activation of innate and/or adaptive immune responses with CpG ODNs has proven to have great promise for the prevention and therapy for cancer, allergy, and infectious diseases.[4,5]

These results suggest therapeutic potential for CpG ODNs in treating various human diseases, although one must be mindful of the interspecies differences in TLR9 cellular distribution and optimal CpG sequences. A logical first step in this process is to evaluate biomarkers of innate immune activation after administration of CpG ODNs to human volunteers with intact immune systems. In this report we present the safety and immunopharmacology of CPG 7909, a B-class (also called K-type) CpG ODN, administered through either the IV or SC route to healthy human volunteers in two separate phase 1 randomized, placebo-controlled, double-blind trials. These studies show that systemic activation of TLR9 in humans with SC injection of a B-class CpG ODN appears to be safe and induces a T_H1-like cytokine and chemokine profile that is distinct from that induced in mice and is consistent with the known cellular pattern of TLR9 expression.

MATERIALS AND METHODS

CPG 7909

CPG 7909, a B-class CpG ODN of sequence 5′-TCG-TCGTTTTGTCGTTTTGTCGTT-3′, which is optimized for stimulation of human TLR9, was synthesized by GMP with a wholly phosphorothioate backbone (Coley Pharmaceutical Group, Wellesley MA) and had undetectable levels of endotoxin. The drug was dissolved in phosphate-buffered saline at a final concentration of 20 mg/mL per vial.

Study Designs and Procedures

Two separate clinical trials were conducted to investigate the safety and immune effects of CPG 7909, one testing the SC route and the other the IV route of administration to healthy men aged 18 to 45 years. Both trials were phase 1 randomized, double-blind, placebo-controlled, dose-escalation single-center studies, carried out at the FOCUS Clinical Drug Development GmbH, Germany. The studies were reviewed and approved by the Independent Ethics Committee of the Ärztekammer Nordrhein.

The SC study enrolled six consecutive overlapping cohorts of subjects with rising doses of CPG 7909 (0.0025, 0.005, 0.01, 0.02, 0.04, and 0.08 mg/kg). Each cohort of eight subjects comprised six randomized to receive CPG 7909 and two receiving the physiologic saline placebo. Each subject was to receive two injections of the same dose level, separated by an interval of 14 days, with follow-up to 58 days after the first injection. Safety and tolerability endpoints included adverse events (AEs), clinical laboratory evaluations (hematology, clinical chemistry, urinalysis, and coagulation), assessment for swelling of spleen or lymph nodes, vital signs measurement, a 12-lead ECG, and a 24-hour Holter ECG. In addition, immune status parameters including ANA, anti-dsDNA, RF, C3, C4, and CH-100 were assessed. Serum samples were collected for immune pharmacodynamic assays as indicated in Tables 1 and 2.

Toxicity was assessed by the National Cancer Institute (NCI) Common Toxicity Criteria (CTC), version 2.0. The relationship to treatment was recorded by the investigator based on the NCI 5-point scale (unrelated, unlikely, possible, probably, or definitely). Toxicities of grade 3 or higher that could not be immediately assessed as unrelated to study drug were generally a reason for concern and automatically required postponement of the next higher dosing group.

The IV safety study was performed separately but was designed similarly to the SC study, with the exceptions that the dose levels were different and went higher (0.001, 0.005, 0.01, 0.04, 0.16, and 0.32 mg/kg) and that the second dose was given 7 rather than 14 days after the first. The doses were given as an IV infusion over 2 hours. Monitoring for AEs and immune reactions was performed as in the SC trial.

Pharmacodynamic Assays

Frozen serum samples were shipped on dry ice to Coley Pharmaceutical Canada. Upon receipt, sample identity was verified and the integrity of the samples assessed; samples were then stored at −70° to −85°C until analysis. To maintain blinding, samples were identified by subject numbers and initials only. Before analysis, serum samples were aliquoted such that each aliquot would be thawed a maximum of two times before being analyzed. Due to the large number of cytokine and chemokine assays being performed and the limited amount of serum available, samples were serially diluted two-fold before analysis, resulting in the following sample dilutions 1:2 and 1:4 for the following analyses: IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-12p40, IL-18, IFN-γ, IFN-α, TNF-α, IP-10, MCP-1, MIP-1α, and MIP-1β. When available, commercial kits were used and tests were performed according to the manufacturer's instructions. The following commercial ELISA kits for quantification of chemokines or cytokines in human serum were purchased from R&D Systems, Inc. (Minneapolis, MN): IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-12p40, IL-18, TNF-α, IFN-γ, MCP-1, MIP-1α, and MIP-1β. The IFN-α ELISA kit was purchased from PBL-Biomedical Laboratories (New Brunswick, NJ), the -CRP ELISA kit from Alpco Diagnostics (Windham, NH), and the RF ELISA Kit from Zeus Scientific Inc (Raritan, NJ).

ELISA assays were developed and optimized for the detection of MIG in human serum using development reagents from BD Biosciences Pharmingen (San Diego, CA) and MIP-3β and IGFBP-4 using development reagents from R&D Systems, Inc. The assays were performed according to the manufacturer's directions. 2′5a synthetase activity was determined using a commercially available radioimmunoassay from Eiken Chemical Company, Ltd (Tokyo, Japan), according to the manufacturer's instructions.

Coley Pharmaceutical Group's Clinical Immune Assay Laboratory developed the IP-10, I-TAC, total IgG, IgG1, IgG2, IgG3, IgG4, IgA, IgM, and IgE ELISA assays. The

TABLE 1. Assay Methods

ELISA	Primary Antibody	Standard	Standards Concentrations	Sample Volume (µL)	Sample Dilution	Controls	Secondary Antibody
IP-10	0.5 µg/mL, anti-human IP-10 (BD Biosciences Pharmingen) in carbonate buffer (Sigma-Aldrich, Inc. Oakville, ON)	Human IP-10 (BD Biosciences Pharmingen)	19.5 to 1,250 pg/mL	100	1/2 and 1/4	Human serum alone	Biotin-labeled mouse anti-human IP-10 (BD Biosciences Pharmingen) diluted 1/8,000
I-TAC	0.5 µg/mL, anti-human I-TAC (R&D Systems, Inc) in phosphate buffered saline (Mediatech, Inc, Herndon, VA)	Human I-TAC (R&D Systems, Inc)	19.5 to 1,250 pg/mL	100	1/2 and 1/4	Human serum alone	Biotin-labeled goat anti-human I-TAC (R&D Systems, Inc) diluted 1/500
IgG	2 µg/mL, mouse anti-human IgG (BD Biosciences Pharmingen) in carbonate buffer (Sigma-Aldrich, Inc)	Human IgG (Sigma-Aldrich, Inc)	1.95 to 250 ng/mL	50	1/8,000 to 1/8,192,000	Human serum alone, human serum minus IgG, IgA and IgM (Sigma-Aldrich, Inc)	HRP-labeled anti-human IgG (Southern Biotechnology Associates Inc)
IgG1	10 µg/mL, mouse anti-human IgG1 (BD Biosciences Pharmingen) in carbonate buffer (Sigma-Aldrich, Inc)	Human IgG1 (Sigma-Aldrich, Inc)	1.95 to 250 ng/mL	50	1/8,000 to 1/8,192,000	Human serum alone, human serum minus IgG, IgA and IgM (Sigma-Aldrich, Inc)	HRP-labeled anti-human IgG (Southern Biotechnology Associates Inc)
IgG2	10 µg/mL, mouse anti-human IgG2 (Southern Biotechnology Associates Inc, Birmingham, AL) in carbonate buffer (Sigma-Aldrich, Inc)	Human IgG2 (Sigma-Aldrich, Inc)	1.95 to 250 ng/mL	50	1/500 to 1/128,000	Human serum alone, human serum minus IgG, IgA and IgM (Sigma-Aldrich, Inc)	HRP-labeled anti-human IgG (Southern Biotechnology Associates Inc)
IgG3	10 µg/mL, mouse anti-human IgG3 (BD Biosciences Pharmingen) in carbonate buffer (Sigma-Aldrich, Inc)	Human IgG3 (Sigma-Aldrich, Inc)	3.9 to 500 pg/mL	50	1/500 to 1/8,000	Human serum alone, human serum minus IgG, IgA and IgM (Sigma-Aldrich, Inc)	HRP-labeled anti-human IgG (Southern Biotechnology Associates Inc)
IgG4	10 µg/mL, mouse anti-human IgG4 (BD Biosciences Pharmingen) in carbonate buffer (Sigma-Aldrich, Inc)	Human IgG4 (Sigma-Aldrich, Inc)	1.95 to 250 ng/mL	50	1/500 to 1/128,000	Human serum alone, human serum minus IgG, IgA and IgM (Sigma-Aldrich, Inc)	HRP-labeled anti-human IgG (Southern Biotechnology Associates Inc)
IgA	10 µg/mL, mouse anti-human IgA (BD Biosciences Pharmingen) in carbonate buffer (Sigma-Aldrich, Inc)	Human IgA (Calbiochem Novabiochem, San Diego, CA)	1.95 to 125 ng/mL	50	1/80,000 to 1/640,000	Human serum alone, human serum minus IgG, IgA and IgM (Sigma-Aldrich, Inc)	Biotin-labeled anti-human IgA (BD Biosciences Pharmingen)
IgM	4 µg/mL, mouse anti-human IgM (BD Biosciences Pharmingen) in carbonate buffer (Sigma-Aldrich, Inc)	Human IgM (Sigma-Aldrich, Inc)	3.9 to 250 ng/mL	50	1/10,000 to 1/80,000	Human serum alone, human serum minus IgG, IgA and IgM (Sigma-Aldrich, Inc)	Biotin-labeled anti-human IgM (BD Biosciences Pharmingen)
IgE	4 µg/mL, mouse anti-human IgE (Southern Biotechnology) in carbonate buffer (Sigma-Aldrich, Inc)	Human IgE (Calbiochem)	0.49 to 62.5 ng/mL	50	1/2 to 1/16	Human serum alone	HRP-labeled anti-human IgE (Southern Biotechnology)

assays were performed according to the standard method outlined below, with the specific details such as primary and secondary antibodies used as outlined in Table 1. Briefly, 96-well plates (Corning Costar, Acton, MA) were coated overnight at 4°C with the appropriate primary antibody. Following the overnight incubation, plates were washed such that each well was washed five times with at least 200 µL of wash buffer (PBS + 0.05% Tween20 [Sigma-Aldrich, Inc]). The plates were subsequently blocked for 1 to 2 hours with at least 200 µL blocking buffer (PBS + 0.05% Tween20 + 10% FBS [Gibco Invitrogen Corp, Burlington, ON]) per well. Following the 1-hour incubation, the plates were washed and the appropriate standards were added to each plate in duplicate. Human serum samples serially diluted in dilution

TABLE 2. Summary of Immune Assay Results

Assay	Doses Tested*	Time Points Tested†	Result‡
Cytokines			
IL-1beta	0.0 (n = 1); 0.04 (n = 1) and 0.08 mg/kg (n = 6)	All	<0.25 pg/mL
IL-2	All	All	<62.5 pg/mL
IL-4	All except 0.0 (n = 2) and 0.04 mg/kg (n = 6)	All	<0.5 pg/mL
IL-6	All	All	+
IL-8	0.0 (n = 1) and 0.04 mg/kg (n = 1)	All	−
IL-10	All	All	<15.6 pg/mL
IL-12p70	All	All	<1.562 pg/mL
IL-12p40	All	All	+
IL-18	All	All	−
IFN-alpha	All	All	+
IFN-gamma	All except 0.0 (n = 2) and 0.04 mg/kg (n = 6)	All	<31.25 pg/mL
TNF-alpha	All	All	−§
Chemokines			
IP-10	All	All	+
I-TAC	0.0 (n = 4), 0.0025 (n = 3), 0.02 (n = 2), 0.08 mg/kg (n = 5)	All	+
MCP-1	All	Pre-dose, 12, 18 and 24 hours post-dose	+
MIG	0.0 (n = 1) and 0.08 mg/kg (n = 5)	All	+
MIP-1alpha	0.08 mg/kg (n = 4)		<93.8 pg/mL
MIP-1beta	All	All	+
MIP-3beta	0.0 (n = 1) and 0.08 mg/kg (n = 5)	All	+
Immunoglobulin Isotypes			
IgG, 1, 2, 3, 4	All	All	−
IgA, IgM, IgE	All	All	−
Other			
C-reactive protein	All	All	+
Rheumatoid factor	All	Pre-dose and last visit (day 58)	−
IGFBP-4	0.0 (n = 1) and 0.08 mg/kg (n = 3)	All	−
2'5a synthetase	0.0 (n = 5), 0.0025 (n = 2), 0.005 (n = 2), 0.01 (n = 2), 0.02 (n = 2) and 0.04 mg/kg (n = 2)	Pre-dose, 12 or 18, 24, 48 hours post-dose and last visit (day 58)	+

*"All" indicates that these parameters were tested in serum samples collected from each dose group (0.0–0.08 mg/kg).

†"All" indicates that samples were assessed at 0, 1.5, 3, 12, 24 (day 2 or 16), 48 (day 3 or 17) and 144 (day 7 or 21) hours after the first and second administration and end of treatment (~day 58) for all dose groups, and additionally at 2 and 18 hours post-dose for the highest dose group (0.08 mg/kg).

‡Indicates whether there was a CpG-induced change from baseline (pre-dose) in this parameter to at least twice the normal level for that assay. *<value* indicates that the majority of results were less than the lower limit of quantification shown.

§Serum TNF-α levels showed a slight trend to dose-dependent increase at the two highest dose levels, but the peak serum values at 24–48 hours averaged less than a two-fold increase above the normal range for this assay and therefore did not meet our criteria for positive.

buffer were also added to each plate, along with duplicate blank wells containing dilution buffer alone and appropriate controls. The plates were incubated at room temperature for 2 hours followed by washing. Diluted biotin-labeled or HRP-labeled secondary antibody was added to each well and the plate was left to incubate for 1 hour at room temperature. For assays using biotin-labeled antibody, the plates were washed and avidin-peroxidase (Sigma-Aldrich, Inc) diluted 1:1,000 was added to each well and the plates were incubated for 30 minutes. Subsequently for all ELISA assays, the plates were washed and then TMB Liquid Substrate (Sigma-Aldrich, Inc) was added to each well. The plates were placed in the dark at room temperature for 30 minutes; following the incubation, 50 μL of 2N sulfuric acid was added to each well. Absorbance readings for each well were determined using a microplate reader. Duplicate readings for each sample and standard were averaged and the average blank reading subtracted. Concentrations of each analyte were interpolated from the standard curve and multiplied by the appropriate dilution factor. Samples below the lowest standard were considered out of range and recorded as less than the lowest standard times the dilution factor. Samples that had absorbance readings higher than that of the highest standard were further diluted as required and reassessed as outlined above.

Data Analysis and Statistical Evaluations

The analyses for safety and tolerability parameters were performed using the safety population, which was defined as all randomized subjects who received at least one dose of study medication and had post-dose safety information. Pharmacodynamic parameters were analyzed using the intent-to-treat population. For categorical variables, the number and percentage of subjects in each category were summarized. Continuous variables were summarized by the number of

observations, mean, standard deviation, median, minimum, and maximum. Missing data were reflected by varying sample sizes across visits and was not imputed. All statistical analyses were performed using SAS version 8.2. Graphs were generated that show the mean changes from baseline for pharmacodynamic parameters over time by dose levels. Linear correlations between parameters of interest were generated using linear regression analyses.

RESULTS

Safety and Tolerability of SC and IV CPG 7909

SC Study

For the SC study, 56 subjects were planned and 57 were randomized; 56 subjects were analyzed for safety and pharmacodynamics. Thirteen subjects were assigned to the placebo group and 43 received at least one dose of CPG 7909; six were assigned to each of the 0.0025-, 0.005-, 0.01-, single-dose 0.01-, 0.02-, and 0.08-mg/kg groups and seven were assigned to the 0.04-mg/kg group. The study was initiated with a 0.01-mg/kg cohort, which was planned as a no-effect level. After the first injection, six of the eight subjects in that cohort reported AEs. These subjects did not receive the second injection of CPG 7909 (single-dose 0.01-mg/kg) and were classified as prematurely terminated from the study. To identify a no-effect level, or at least close to no effect, the protocol was amended to restart the study using lower doses of CPG 7909.

Almost all (91%) of subjects in the SC study reported AEs. While the incidence of AEs was similar among most dose groups, the actual number of events reported was higher in the higher active dose groups (0.02, 0.04, and 0.08 mg/kg). Eighty-three events were reported in the 0.08-mg/kg group compared with 18 in the placebo group. The 0.02- and 0.04-mg/kg groups experienced 56 events each. Most of the AEs were mild in severity; only a few were classified as moderate. To better characterize the tolerability profile of CPG 7909, composite scores for local injection site reactions and flu-like symptoms were developed (Fig. 1). Overall, there appeared to be a possible trend for AEs to be reported more commonly after the first injection than the second, especially at the lower dose levels. The two lowest-dose groups of SC CPG 7909 showed no apparent increase in systemic symptoms compared with the subjects receiving placebo, despite showing some injection site reactions, which were not reported by any of the placebo subjects.

The most commonly reported injection site AEs were injection site erythema, occurring in 29 of the 56 (52%) subjects overall, and injection site pain, occurring in 24 of the 56 (43%) subjects. Other injection site AEs included induration (30%), edema (18%), pruritus (13%), and inflammation (7%). Flu-like symptoms were less commonly reported and were generally described as mild. The maximum febrile responses were dose-related, with increases up to 39.2°C.

In summary, SC injection of CPG 7909 induced dose-dependent clinical signs of immune stimulation (local reactions, flu-like symptoms), but no clinically significant toxicities occurred with doses up to 0.08 mg/kg.

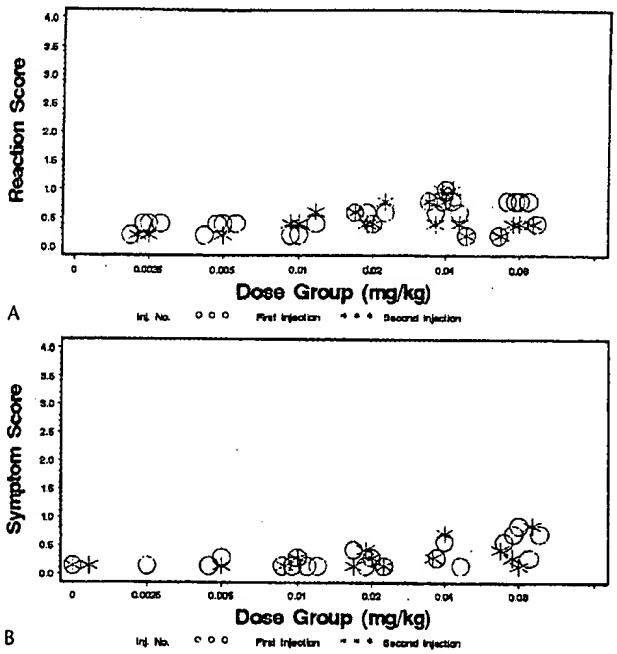

FIGURE 1. AEs reported after SC injection of CPG 7909. A, Local tolerability composite index. Each local symptom (pain, erythema, warmth, edema, and inflammation) was graded 0, 1, 2, 3, or 4, for absent, mild, moderate, severe, or life-threatening and then summed and divided by 5 for each subject after the first injection (open circles) or second injection (stars). Subjects who reported no AEs are not shown. The results of this composite index are presented by dose level (dose group 0 are placebo subjects). B, Systemic tolerability composite index. Reports of fever, chill, myalgias, arthralgias, fatigue, headache, and musculoskeletal pain were assigned a numeric grade (see above) and were then summed and divided by 7.

IV Study

For the IV study, 50 subjects were randomized in six consecutive cohorts with rising doses of CPG 7909 (0.001, 0.005, 0.01, 0.04, 0.16, and 0.32 mg/kg). Each cohort comprised eight subjects (six receiving CPG 7909 and two receiving placebo). Overall, 83 AEs were experienced by 30 (60%) subjects during the study. The incidence of AEs ranged from 33% (0.01 mg/kg) to 83% (placebo). There was no dose-dependent frequency of AEs, and the types of AEs were not different in the sequential dose groups. The most commonly reported AEs included lymphadenopathy, headache, and nasopharyngitis, most evident in subjects receiving placebo, but with an increasing frequency of lymphadenopathy with higher doses of CPG 7909. However, the number of subjects experiencing lymphadenopathy in the 0.04-mg/kg to the 0.32-mg/kg groups was similar to that observed in subjects in the placebo group. Reactions at the IV infusion sites were rare and of minimal intensity. Local injection site reactions were extremely rare and appeared to be associated in one incidence with local extravasation of the IV infusion. There were no reports of thrombophlebitis or vascular sclerosis in association with IV infusion. Systemic, flu-like symptoms were slightly

more frequent in the CPG 7909 groups than the placebo group and were mild.

Overall, CPG 7909 by the IV route was associated with very low local and systemic reaction scores, similar to placebo.

Induction of Serum Cytokines and Chemokines After SC CPG 7909

Serum samples were collected from the normal volunteers receiving SC injections with either saline or CPG 7909 at the time points as indicated on Table 2 and were analyzed in a blinded fashion. To determine whether there was any sensitization or tolerance to repeat injection with the CPG 7909, a second injection was given 2 weeks after the first and sera were collected from the subjects at the same times as after the first injection. To assess fully the pharmacodynamic effects of SC CPG 7909, a wide range of immune assays were performed on these sera. In total, more than 30 different immune assays were performed on approximately 1,300 serum samples collected in this and the companion IV study (see below).

The results of all of the immune assays for the SC clinical trial are summarized in Table 2. For most of the cytokines tested, there was no statistically significant change at any dose level of CPG 7909. Likewise, there was no change in serum Ig levels or isotypes at any dose or time point. However, at one or more dose levels there was an increase in the serum level of all of the tested chemokines except for MIP-1α, which was uniformly below the level of detection of the assay. The most sensitive of all of the assays used in the trial was the IP-10, which showed statistically significant increases at every dose level of SC CPG 7909, even the lowest dose of just 0.0025 mg/kg. The mean serum IP-10 levels showed a dose-dependent increase in response to SC CPG 7909, with kinetic analyses demonstrating a detectable increase as early as 12 hours after injection and a peak level at approximately 24 hours, with a gradual decrease back toward baseline (Fig. 2A). At the high dose levels, serum IP-10 levels were still significantly elevated above baseline 7 days after the dose.

Serum IL-6 and MCP-1 levels showed dose-dependent increases with similar kinetics to IP-10 but with less sensitivity, as the increases were not statistically significant at the lowest dose levels (see Figs. 2B,C). Serum CRP levels also increased in a dose-dependent fashion but with slower kinetics, the peak occurring approximately 48 hours after CpG injection and remaining elevated at 7 days post-dosing (see Fig. 2D).

IP-10 is an IFN-inducible chemokine. Surprisingly, no increase above the sensitivity of the kit in serum IFN-γ or IFN-α could be detected after SC CPG 7909 at doses as high

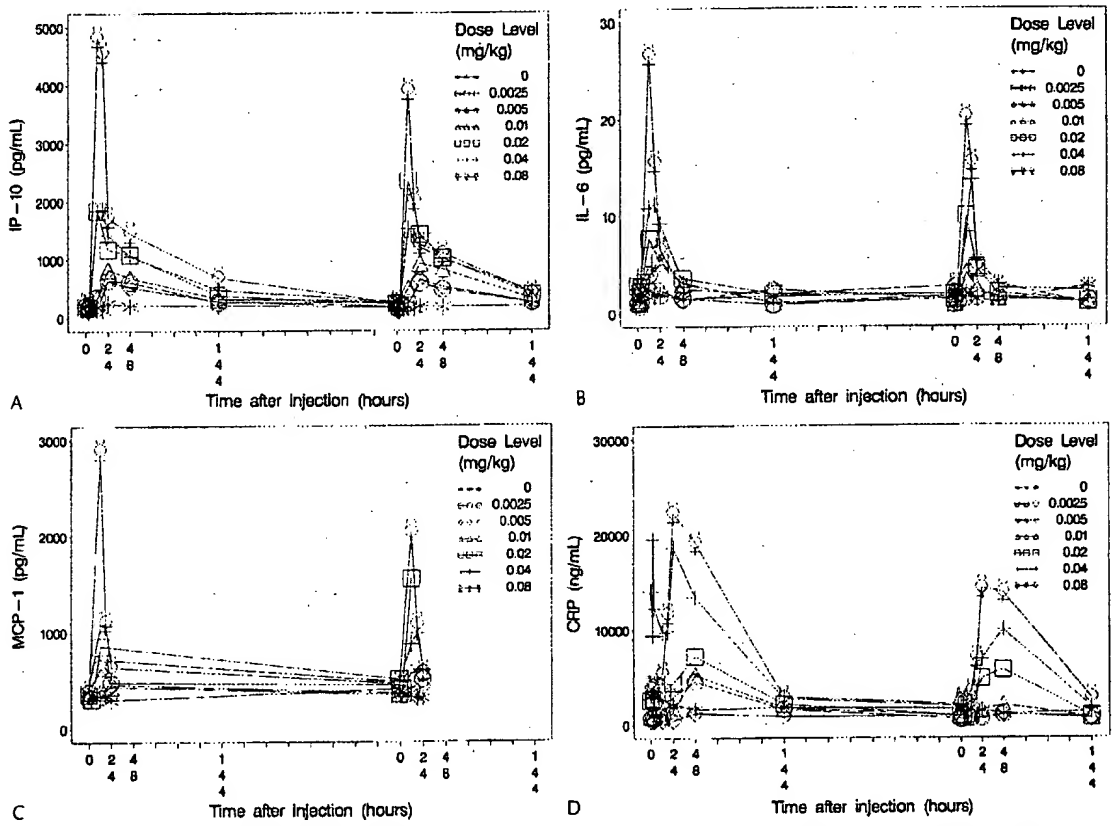

FIGURE 2. Kinetics of serum markers for innate immune activation and acute phase response by SC CPG 7909. Sera from all subjects in the SC trial were collected at the time points shown in Table 2 and assayed by ELISA for IP-10 (A), IL-6 (B), MCP-1 (C), and CRP (D). The data points indicate the mean values for the six subjects at each dose level of CPG 7909. The values for the subjects who received placebo were combined from each cohort and plotted together as one group.

as 0.04 mg/kg, despite the large and sustained increases in serum IP-10. A small increase in serum IFN-α was detected at the highest dose level of 0.08 mg/kg (Fig. 3A). The kinetics of this IFN-α response were slightly faster than the IP-10, with a peak at 12 hours and return to baseline by 24 hours, consistent with previous in vitro reports that IFN-α induces IP-10 secretion.[18] There was a noticeable tendency toward a biphasic pattern to the serum IFN-α levels, with a second lower peak in several subjects at day 3.

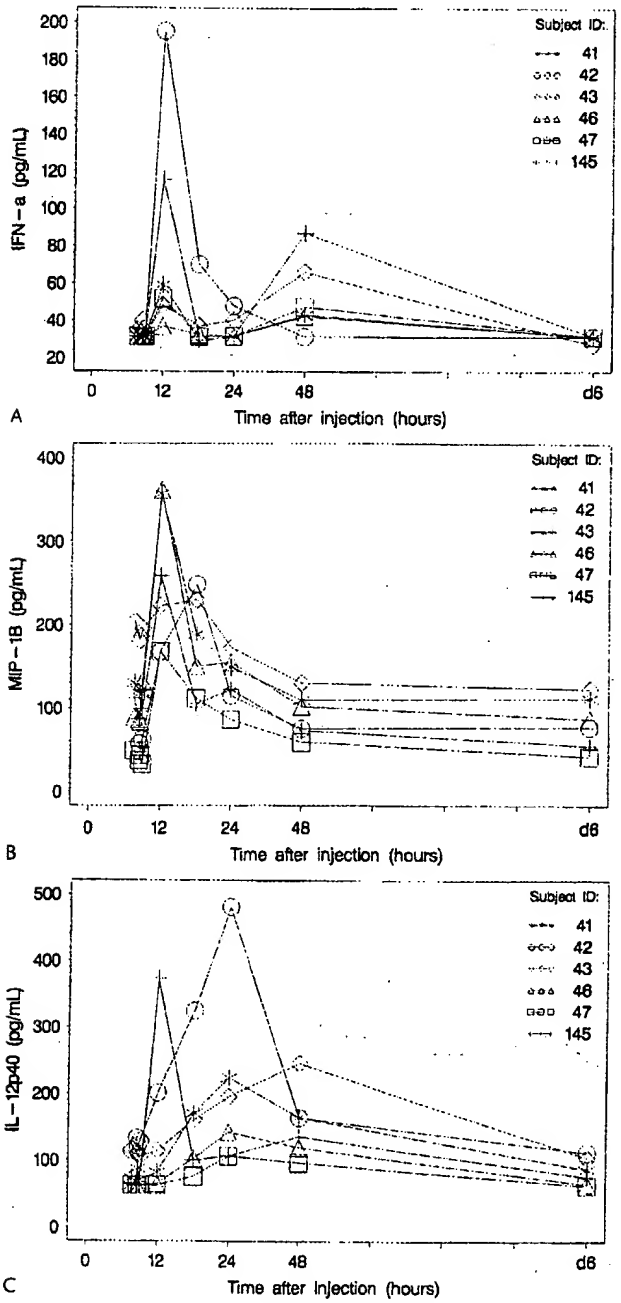

FIGURE 3. Kinetics of serum responses to SC CPG 7909 at 0.08 mg/kg. Significant increases in serum IFN-a, MIP-1b, and IL-12p40 were seen only at the highest SC dose level, so the results with the lower doses are not shown. To show the subject-to-subject variability in responses, each line represents the values for an individual subject after the first dose.

Despite the failure to detect serum IFN-α at any but the highest dose of CPG 7909, in vitro studies have documented clearly the ability of CPG 7909 to induce human pDCs to secrete IFN-α.[22] Therefore, we considered it possible that IFN-α was being produced and was locally consumed in the SC tissues or draining lymph nodes. To determine whether other IFN-inducible factors were also present in the CpG-treated subjects, we tested selected sera for MIP-1α, MIP-1β, MIP-3β, 2'5' OAS, I-TAC, and MIG. For several of these factors we found increases after CpG injection, confirming the induction of an IFN immune profile (see Fig. 3B for MIP-1β, and Table 2). IP-10 binds to the receptor CXCR3, which is also bound by the IFN-inducible genes I-TAC and MIG. We found increases in the serum levels of I-TAC and MIG at the same time points as IP-10, but the fold increases were substantially less, indicating that IP-10 may be the most sensitive marker for the IFN effect induced through in vivo TLR9 stimulation by CPG 7909.

The only other factor to show significant increases in most subjects was IL-12p40, which increased in all subjects 12 to 24 hours after SC CPG 7909 (see Fig. 3C).

Interindividual Variability in Responses to SC CPG 7909

Every subject showed an increase in serum IP-10 levels after SC CPG 7909, regardless of the dose level (Fig. 4). There was a tendency to higher mean peak levels with higher CPG 7909 doses, although there was much overlap between individuals in the dose groups. Within individual mg/kg dose groups, the peak serum IP-10 level did not appear to correlate with absolute dose, suggesting that it is appropriate in clinical trials to dose CPG 7909 based on body weight. At CPG 7909 doses up to 0.02 mg/kg, the serum IP-10 response appeared similar after both the first and second doses. However, at the two highest CPG 7909 dose levels, there was a tendency for those subjects with the highest IP-10 levels after the first dose to have a slightly lower serum IP-10 after the second dose. These data suggest the possibility that repeat SC administration of CPG 7909 at high doses may result in a slight decrease in the level of innate immune activation, particularly if the initial response was very strong. There was no significant difference in the kinetics of the immune responses between the first and second doses.

Correlation Between Serum IL-6 and IP-10 Responses to SC CPG 7909

Since the only cells in humans that are known to express TLR9 are B cells and pDCs, the immune effects of CPG 7909 must result directly or indirectly from these cell types. IP-10 production is thought to result from pDC activation,[18] while B-cell stimulation has been reported to lead to IL-6 expression.[4] IP-10 is known to be regulated in an IFN-dependent manner, whereas IL-6 is not. Thus, it was unclear whether in individual patients the levels of these markers would correlate. When the peak serum levels for both markers in individual

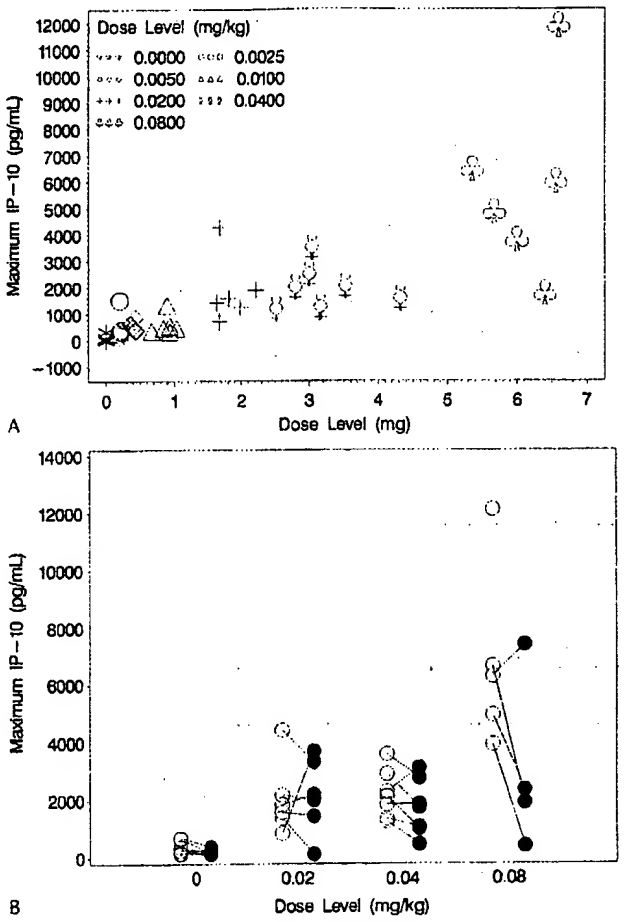

FIGURE 4. Inter- and intra-individual variability in IP-10 response to SC CPG 7909. A, Peak serum IP-10 after the first injection of SC CPG 7909 is plotted compared with the absolute dose that the patient was actually given on the x-axis. B, To show possible dose-to-dose differences in the level of IP-10 response, the peak serum IP-10 at any time point after both doses is plotted, with a line connecting the results for each individual subject (open circle, first dose; closed circle, second dose). Because there was much less variability at the lower dose levels, only the placebo and three highest CPG 7909 groups are shown.

patients were plotted together, there was a significant correlation (R = 0.55), showing that subjects who had a high (or low) IL-6 response to CPG 7909 also tended to have a relatively high (or low) response in IP-10 secretion (Fig. 5).

Failure of IV CPG 7909 to Increase Serum Cytokines or Chemokines

Even though the clinical trial administering CPG 7909 through the IV route went up to doses four times higher than the highest dose given in the SC trial, surprisingly there was no significant change in any of the immune assays at any of the tested time points (not shown).

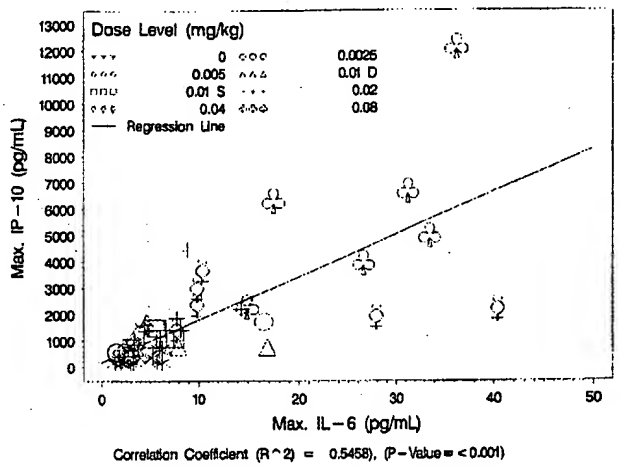

FIGURE 5. Correlation between serum IL-6 and IP-10 responses to SC CPG 7909 injection. Even within a single dose cohort, there was substantial variability between subjects in the magnitude of the serum cytokine and chemokine responses. However, a significant association was noted between peak serum IP-10 and IL-6 levels in subjects after receiving SC CPG 7909.

Redistribution of Leukocytes in Response to SC CPG 7909

IP-10 and other chemokines whose expression is induced by SC CPG 7909 are known to alter the immune cell trafficking. For example, CXCR3, the receptor for IP-10, is expressed on activated T cells, B cells, and NK cells. Human neutrophils may express several chemokine receptors, including CCR1, CCR4, CXCR1, CXCR2, and CXCR4.[23] These data suggested the possibility that CpG-induced chemokines could cause a transient redistribution of some cell types into or out of the blood. Indeed, as shown in Figure 6, blood neutrophil levels showed a biphasic response to CPG 7909 injection, with an early increase at 12 hours, followed by decreased levels for at least 72 hours, followed by a return to baseline before the second injection was given. The lowest absolute neutrophil count observed was 800 cells/μL, which rebounded above 1,000 cells/μL within 48 hours. No infections or other clinical evidence for immune suppression occurred in conjunction with these transient episodes of neutropenia. The absolute lymphocyte count showed a sharp drop at 12 hours after CPG 7909 injection but returned to baseline by 48 hours. In contrast, the absolute blood monocyte count was increased at 24 hours after injection, with a gradual return toward baseline over the following 48 hours.

IV administration of CPG 7909 caused no redistribution of immune cells at the dose levels tested, consistent with the lack of induction of chemokine expression (not shown).

Lack of Evidence for Induction of Autoimmunity

The immune status of study subjects was monitored at the start and end of the study with assays for antinuclear antibody, anti-dsDNA, rheumatoid factor, C3, C4, and CH-100. Although values from some subjects were outside the normal range both at baseline and at subsequent time points, there was no study-related change in these parameters.

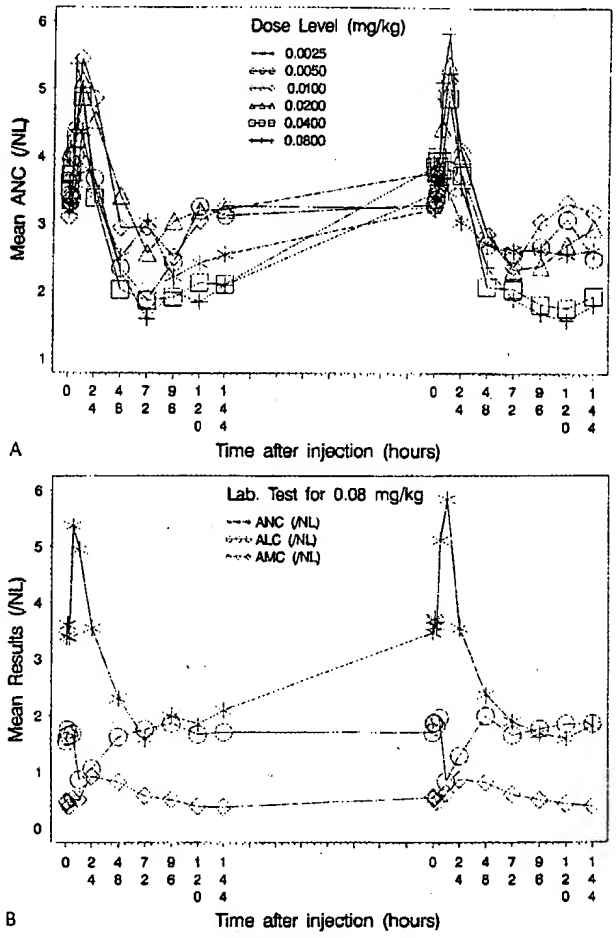

FIGURE 6. Kinetics of CPG 7909-induced leukocyte redistributions. A, Dose-dependent CPG 7909-induced neutrophil redistribution. The group mean absolute neutrophil counts are plotted for each of the CPG 7909 dose groups and the controls at each of the tested time points after the first and second injection. B, Kinetics of neutrophil, lymphocyte, and monocyte redistributions after SC CPG 7909. The mean absolute neutrophil, lymphocyte, and monocyte counts are plotted for the high-dose CPG 7909 group at each of the tested time points after the first and second injection.

DISCUSSION

Our results show that SC injection of CPG 7909 in normal human volunteers induces a rapid systemic T_H1-like chemokine and cytokine response that is dose-dependent and remarkably sensitive. Indeed, we did not identify a no-response dose for CPG 7909 in humans: even the lowest tested dose of 0.0025 mg/kg (approximately 175 μg), which is close in absolute terms to therapeutic CpG ODN doses used in mouse immunotherapy models, induced an average three-fold increase from the baseline serum IP-10 levels. The systemic type I IFN response is characterized by increased serum levels of multiple IFN-inducible genes, including IP-10, I-TAC, MIG, MIP-1β, and MIP-3β. Serum IFN-α levels were increased at the highest dose levels. This finding is consistent with the in vivo stimulation of pDCs, which express the TLR9 receptor for CPG 7909. pDCs secrete type I IFN in response to in vitro stimulation with CpG ODNs,[24] resulting in the secretion of IP-10 from both pDCs and from bystander IFN-responsive cells such as monocytes.[18,22] Our results show that this same pattern of T_H1-like innate immune activation response can be detected in vivo, and that it includes multiple IFN-inducible genes.

The innate immune effects of CPG 7909 injection in humans are markedly different from those reported previously in mice. Mice injected with CpG ODNs show strong induction of serum TNF-α, IL-6, IL-12, and IFN-γ.[19,20,25] In contrast, only serum IL-6 and IL-12p40 showed significant increases in our human trial, and these increases were relatively lower in magnitude compared with those previously reported in mice. We propose that the different serum cytokine profiles resulting from CPG 7909 administration to humans and mice can be explained by the much more restricted expression of TLR9 in human as compared with mouse immune cells.[7–9,26] In normal primary human cells, functional TLR9 expression is only confirmed in pDCs and B cells, but mice also express TLR9 in their monocytes, macrophages, and myeloid DCs.[10,11] The kinetics of these responses also differ: in mice the peak serum chemokine and cytokine responses are at 3 to 4 hours, while in humans the response is undetectable until 12 hours and peaks at approximately 24 hours.

High systemic levels of the proinflammatory cytokines expressed in CpG ODN-injected rodents have been associated with a broad range of adverse effects. Indeed, very high CpG doses given by intraperitoneal injection daily for 20 days recently were reported to lead to severe toxicity, including multifocal hepatic necrosis and destruction of lymphoid follicles in mice.[27] Previous studies showed that high-dose CpG ODN treatment in rodents can cause extramedullary hematopoiesis and an IFN-γ and TNF-associated lethal systemic inflammatory response syndrome (SIRS).[28–31] Extramedullary hematopoiesis and fatal SIRS can be induced in rodents injected with high-dose CPG 7909 but have not been observed in primates, even after twice-weekly administration at 5 mg/kg for 6 months (Coley Pharmaceutical Group, data on file). We found no abnormality in serum liver function tests, change in serum Ig levels, or evidence for other clinically significant toxicities in humans injected with any dose level of CPG 7909 through either the SC or IV route. These results suggest that the toxicities reported in rodents after the administration of CpG ODNs are unlikely to occur in humans or non-human primates, presumably because of the species-specific differences in TLR9 expression. Likewise, therapeutic stimulation of TLR9 in humans may be much better tolerated than stimulation of other TLRs that are expressed on a broader range of immune cells.

TLR9 stimulation with CpG ODNs induces counter-regulatory mechanisms such as downregulation of IRAK that result in a transient decrease in responsiveness at the cellular level.[32] However, no CpG-induced tolerance or tachyphylaxis has been detectable in vivo in rodents[33] (Waldschmidt T, Krieg AM, unpublished results). Our present results show also in humans that a second low-dose SC injection of CPG 7909 leads to a similar pattern and degree of innate

immune activation compared with that seen after an initial injection.

Humans have a tremendous degree of interindividual variability in the degree of innate immune activation to endotoxin exposure. Although the numbers of humans exposed to SC CPG 7909 in our trial was small, we found no evidence for major polymorphisms in the response to TLR9 stimulation. Every subject injected SC with CPG 7909 showed an increase in serum IP-10, even at the lowest dose level of 0.0025 mg/kg. As for individual bioassays, there were interindividual differences in the magnitude of the serum increases in IP-10 and in the other tested pharmacodynamic markers. These responses showed a clear dose-dependent pattern, with overlap in the level of peak responses between adjacent dose groups. Our results are consistent with the finding of limited polymorphisms in the TLR9 gene, none of which are known to have functional consequences.[3,4]

IFN-α improves the development of T-cell memory and enhances cross-priming, thereby triggering strong adaptive T_H1 T-cell responses.[35–39] The therapeutic potential of these T_H1 responses is indicated by the successful use of recombinant IFN-α for vaccination and in the immunotherapy of chronic hepatitis C infection as well as certain cancers. We hypothesize that triggering endogenous production of multiple IFN-α isoforms as well as other type I IFNs through deliberate in vivo activation of the TLR9 pathway could be of considerable value for immunotherapy and vaccination. CPG 7909-induced expression of the full range of endogenous type I IFN genes may be better tolerated and more effective than the administration of pharmacologic doses of a single isoform of exogenous IFN, which may have more restricted immune effects.[40] Moreover, aside from the induction of IFN-α expression, CPG 7909 could have several additional mechanisms of action for immunotherapy. First, the induction of expression of IFN-inducible genes by CPG 7909 appears to be greater than can be explained simply on the basis of IFN-α expression, suggesting the existence of TLR9-induced type I IFN-independent pathways, possibly involving type II IFN.[18] IFN-inducible genes such as IP-10 have anti-angiogenic activity and are involved in the migration of T cells into tumors and in the anti-tumor effect of IL-12, suggesting they could mediate some of the therapeutic effects of CPG 7909.[41–43] A second potential type I IFN-independent therapeutic mechanism for CPG 7909 is the activation of immature human pDCs through TLR9 to mature to highly effective antigen-presenting cells that are capable of inducing primary CTL responses.[22,44–46] Finally, recent studies have shown the ability of TLR9 stimulation to overcome the negative regulatory influence of Treg cells, resulting in stronger therapeutic T-cell responses or reactivation of anergic T cells.[47–50] The current results support the scientific rationale for pursuing human clinical trials of SC CPG 7909 for immunotherapy.

Although recombinant IFN-α can have therapeutic activity in several diseases, it also has been shown to induce autoimmunity in some subjects.[51] Cells from patients with systemic lupus erythematosus (SLE) have been reported to produce increased levels of IFN-α, and a role for IFN-α in DC activation and SLE pathogenesis has been suggested.[52,53] Serum IFN-α levels in SLE patients correlate with disease activity, and it has even been proposed to be a therapeutic target.[54,55] Based on these data, the possibility must be considered that induction of endogenous IFN-α production through TLR9 could promote the development of autoimmunity. We found no increase in autoantibody levels in our subjects injected with CPG 7909, and no evidence of autoimmune disease. This good safety profile is consistent with studies showing that the autoimmune induction through TLR9 appears to require immune complexes[56,57] and does not occur after the injection of naked DNA.[58] Of course, we cannot exclude the possibility that long-term systemic therapy with CpG ODN could induce SLE or other autoimmune diseases in susceptible patients.

The data from these phase I studies show a surprising role for the route of administration in determining the immune effects of CPG 7909. In contrast to the remarkable sensitivity of humans to CPG 7909 administered SC, IV infusion of CPG 7909 up to a 128-fold higher dose than the lowest immune stimulatory SC dose caused no change in any of the measured immune assays or clinical parameters. Our pharmacokinetic studies of CPG 7909 show that as has been reported for antisense ODN, IV administration leads to the rapid distribution into the liver, kidney, and spleen, but SC injection is followed by relatively high concentrations of the ODN in the injection site and draining lymph nodes, which are maintained for at least 2 weeks (Davis H et al, in preparation).[59] We hypothesize that the serum chemokine and cytokine responses to CPG 7909 injection result from high ODN concentrations in the injection site and draining lymph nodes, which stimulate local or recruited pDCs and B cells. The lack of apparent immune activation from IV administration indicates that the CpG-induced innate immunity in humans is far less effectively induced by plasma ODNs or by ODNs taken up in the liver, spleen, or kidney.

Chemokines are known to regulate immune cell trafficking. After SC CPG 7909 injection we have shown dramatic increases in the serum levels of several chemokines. We hypothesize that these factors are responsible for the observed changes in blood levels of neutrophils, lymphocytes, and monocytes, but further studies will be required to determine the precise mechanisms for these cell shifts. The rapid reversibility of these changes indicates that they do not result from bone marrow suppression, which would be expected to last longer.

Aside from pDCs, the TLR9 receptor for CPG 7909 is expressed on B cells, which can lead to their stimulation to enter the cell cycle and to secrete Ig.[3,14] In fact, based on the observation that CpG ODNs can induce human memory B cells to differentiate into Ig-secreting cells in vitro, it has been proposed that polyclonal memory B-cell stimulation by CpG motifs could be important in maintaining serologic memory.[60–63] However, we found that serum Ig levels are unchanged after two injections of CPG 7909, showing that in vivo TLR9 stimulation with CPG 7909 alone at these concentrations is insufficient to induce substantial changes in B-cell homeostasis.

GMP production of CpG ODNs is well established and highly economical. ODNs are produced in bulk (Kg scale) by a solid phase synthesis. Stability of the bulk product appears to

be at least several years when stored frozen; other ODNs have been reported to be stable for at least 2 years even at room temperature. Aqueous solubility is excellent, and the ODNs are relatively nonreactive and nonpyrogenic.

This study was performed with a single CpG ODN, but we and others have reported three distinct classes of CpG ODNs based on differences in their structures and immune effects.[5,64–66] A-class (also called D-type) CpG ODNs induce the production of high levels of IFN-α and marked NK cell activation, with relatively little IL-6 or B-cell stimulation.[61] In contrast, B-class (also called K-type) CpG ODNs, as used in this study, induce the production of modest levels of IFN-α, with much weaker NK-cell activation but with profound B-cell activation.[26] C-class ODNs have intermediate immune effects.[5,65,66] None of the three ODN classes have immune stimulatory activity in mice genetically deficient in TLR9, indicating that TLR9 is required for all of their CpG-induced immune effects.[64] B-class CpG ODNs are superb vaccine adjuvants in mice, primates, and humans for inducing strong antigen-specific immune responses, but relatively little has been published to date on their in vivo immune effects in humans[15,67–74] (Cooper et al, submitted; Speiser et al, submitted). Further studies will be required to determine the in vivo activity of the A- and C-classes in humans.

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REFERENCES

1. Janeway CA Jr, Medzhitov R. Innate immune recognition. *Annu Rev Immunol.* 2002;20:197–216.
2. Takeda K, Kaisho T, Akira S. Toll-like receptors. *Annu Rev Immunol.* 2003;21:335–376.
3. Krieg AM, Yi AK, Matson S, et al. CpG motifs in bacterial DNA trigger direct B-cell activation. *Nature.* 1995;374:546–549.
4. Krieg AM. CpG motifs in bacterial DNA and their immune effects. *Annu Rev Immunol.* 2002;20:709–760.
5. Uhlmann E, Vollmer J. Recent advances in the development of immunostimulatory oligonucleotides. *Curr Opin Drug Discov Dev.* 2003;6: 204–217.
6. Hemmi H, Takeuchi O, Kawai T, et al. A Toll-like receptor recognizes bacterial DNA. *Nature.* 2000;408:740–745.
7. Bauer S, Kirschning CJ, Hacker H, et al. Human TLR9 confers responsiveness to bacterial DNA via species-specific CpG motif recognition. *Proc Natl Acad Sci USA.* 2001;98:9237–9242.
8. Kadowaki N, Ho S, Antonenko S, et al. Subsets of human dendritic cell precursors express different toll-like receptors and respond to different microbial antigens. *J Exp Med.* 2001;194:863–870.
9. Hornung V, Rothenfusser S, Britsch S, et al. Quantitative expression of Toll-like receptor 1-10 mRNA in cellular subsets of human peripheral blood mononuclear cells and sensitivity to CpG oligodeoxynucleotides. *J Immunol.* 2002;168:4531–4537.
10. Dalpke AH, Schafer MK, Frey M, et al. Immunostimulatory CpG-DNA activates murine microglia. *J Immunol.* 2002;168:4854–4863.
11. Pillarisetty VG, Shah AB, Miller G, et al. Liver dendritic cells are less immunogenic than spleen dendritic cells because of differences in subtype composition. *J Immunol.* 2004;172:1009–1017.
12. Rankin R, Pontarollo R, Ioannou X, et al. CpG motif identification for veterinary and laboratory species demonstrates that sequence recognition is highly conserved. *Antisense Nucleic Acid Drug Dev.* 2001;11:333–340.
13. Yi AK, Chang M, Peckham DW, et al. CpG oligodeoxyribonucleotides rescue mature spleen B cells from spontaneous apoptosis and promote cell cycle entry. [published erratum appears in J Immunol 1999 Jul 15; 163(2):1093] *J Immunol.* 1998;160:5898–5906.
14. Hartmann G, Krieg AM. Mechanism and function of a newly identified CpG DNA motif in human primary B cells. *J Immunol.* 2000;164:944–953.
15. Hartmann G, Weeratna RD, Ballas ZK, et al. Delineation of a CpG phosphorothioate oligodeoxynucleotide for activating primate immune responses *in vitro* and *in vivo*. *J Immunol.* 2000;164:1617–1624.
16. Latz E, Schoenemeyer A, Visintin A, et al. TLR9 signals after translocating from the ER to CpG DNA in the lysosome. *Nat Immunol.* 2004; 5:190–198.
17. Ahmad-Nejad P, Hacker H, Rutz M, et al. Bacterial CpG-DNA and lipopolysaccharides activate Toll-like receptors at distinct cellular compartments. *Eur J Immunol.* 2002;32:1958–1968.
18. Blackwell SE, Krieg AM. CpG-A-induced monocyte IFN-gamma-inducible protein-10 production is regulated by plasmacytoid dendritic cell-derived IFN-alpha. *J Immunol.* 2003;170:4061–4068.
19. Klinman DM, Yi AK, Beaucage SL, et al. CpG motifs present in bacterial DNA rapidly induce lymphocytes to secrete interleukin 6, interleukin 12, and interferon γ. *Proc Natl Acad Sci USA.* 1996;93:2879–2883.
20. Yi AK, Klinman DM, Martin TL, et al. Rapid immune activation by CpG motifs in bacterial DNA. Systemic induction of IL-6 transcription through an antioxidant-sensitive pathway. *J Immunol.* 1996;157:5394–5402.
21. Ballas ZK, Rasmussen WL, Krieg AM. Induction of NK activity in murine and human cells by CpG motifs in oligodeoxynucleotides and bacterial DNA. *J Immunol.* 1996;157:1840–1845.
22. Krug A, Rothenfusser S, Hornung V, et al. Identification of CpG oligonucleotide sequences with high induction of IFN-alpha/beta in plasmacytoid dendritic cells. *Eur J Immunol.* 2001;31:2154–2163.
23. Yousefi S, Cooper PR, Potter SL, et al. Cloning and expression analysis of a novel G-protein-coupled receptor selectively expressed on granulocytes. *J Leukoc Biol.* 2001;69:1045–1052.
24. Kadowaki N, Antonenko S, Liu YJ. Distinct CpG DNA and polyinosinic-polycytidylic acid double-stranded RNA, respectively, stimulate CD11c(-) type 2 dendritic cell precursors and CD11c(+) dendritic cells to produce type I IFN. *J Immunol.* 2001;166:2291–2295.
25. Zhao Q, Temsamani J, Zhou RZ, et al. Pattern and kinetics of cytokine production following administration of phosphorothioate oligonucleotides in mice. *Antisense Nucleic Acid Drug Dev.* 1997;7:495–502.
26. Krug A, Towarowski A, Britsch S, et al. Toll-like receptor expression reveals CpG DNA as a unique microbial stimulus for plasmacytoid dendritic cells which synergizes with CD40 ligand to induce high amounts of IL-12. *Eur J Immunol.* 2001;31:3026–3037.
27. Heikenwalder M, Polymenidou M, Junt T, et al. Lymphoid follicle destruction and immunosuppression after repeated CpG oligodeoxynucleotide administration. *Nat Med.* 2004;10:187–192.
28. Cowdery JS, Chace JH, Yi AK, et al. Bacterial DNA induces NK cells to produce IFN-γ in vivo and increases the toxicity of lipopolysaccharides. *J Immunol.* 1996;156:4570–4575.
29. Sparwasser T, Miethke T, Lipford G, et al. Bacterial DNA causes septic shock. *Nature.* 1997;386:336–337.
30. Sparwasser T, Hultner L, Koch ES, et al. Immunostimulatory CpG-oligodeoxynucleotides cause extramedullary murine hemopoiesis. *J Immunol.* 1999;162:2368–2374.
31. Krieg AM. CpG DNA: trigger of sepsis, mediator of protection, or both? *Scand J Infect Dis.* 2003;35:653–659.
32. Yeo SJ, Yoon JG, Hong SC, et al. CpG DNA induces self and cross-hyporesponsiveness of RAW264.7 cells in response to CpG DNA and lipopolysaccharide: alterations in IL-1 receptor-associated kinase expression. *J Immunol.* 2003;170:1052–1061.
33. Klinman DM, Conover J, Coban C. Repeated administration of synthetic oligodeoxynucleotides expressing CpG motifs provides long-term protection against bacterial infection. *Infect Immun.* 1999;67:5658–5663.
34. Lazarus R, Klimecki WT, Raby BA, et al. Single-nucleotide polymorphisms in the Toll-like receptor 9 gene (TLR9): frequencies, pairwise linkage disequilibrium, and haplotypes in three U.S. ethnic groups and exploratory case-control disease association studies. *Genomics.* 2003; 81:85–91.

35. Biron CA, Nguyen KB, Pien GC. Innate immune responses to LCMV infections: natural killer cells and cytokines. *Curr Top Microbiol Immunol.* 2002;263:7–27.
36. Astsaturov I, Petrella T, Bagriacik EU, et al. Amplification of virus-induced antimelanoma T-cell reactivity by high-dose interferon-alpha2b: implications for cancer vaccines. *Clin Cancer Res.* 2003;9:4347–4355.
37. Akbar AN, Lord JM, Salmon M. IFN-alpha and IFN-beta: a link between immune memory and chronic inflammation. *Immunol Today.* 2000;21: 337–342.
38. Le Bon A, Etchart N, Rossmann C, et al. Cross-priming of CD8+ T cells stimulated by virus-induced type I interferon. *Nat Immunol.* 2003;4:1009–1015.
39. Sprent J, Surh CD. T cell memory. *Annu Rev Immunol.* 2002;20:551–579.
40. Hilkens CM, Schlaak JF, Kerr IM. Differential responses to IFN-alpha subtypes in human T cells and dendritic cells. *J Immunol.* 2003;171: 5255–5263.
41. Pertl U, Luster AD, Varki NM, et al. IFN-gamma-inducible protein-10 is essential for the generation of a protective tumor-specific CD8 T cell response induced by single-chain IL-12 gene therapy. *J Immunol.* 2001; 166:6944–6951.
42. Tannenbaum CS, Tubbs R, Armstrong D, et al. The CXC chemokines IP-10 and Mig are necessary for IL-12-mediated regression of the mouse RENCA tumor. *J Immunol.* 1998;161:927–932.
43. Narvaiza I, Mazzolini G, Barajas M, et al. Intratumoral coinjection of two adenoviruses, one encoding the chemokine IFN-gamma-inducible protein-10 and another encoding IL-12, results in marked antitumoral synergy. *J Immunol.* 2000;164:3112–3122.
44. Duramad O, Fearon KL, Chan JH, et al. IL-10 regulates plasmacytoid dendritic cell response to CpG-containing immunostimulatory sequences. *Blood.* 2003;102:4487–4492.
45. Rothenfusser S, Hornung V, Ayyoub M, et al. CpG-A and CpG-B oligonucleotides differentially enhance human peptide-specific primary and memory CD8+ T-cell responses in vitro. *Blood.* 2004;103:2162–2169.
46. Lore K, Betts MR, Brenchley JM, et al. Toll-like receptor ligands modulate dendritic cells to augment cytomegalovirus- and HIV-1-specific T cell responses. *J Immunol.* 2003;171:4320–4328.
47. Serra P, Amrani A, Yamanouchi J, et al. CD40 ligation releases immature dendritic cells from the control of regulatory CD4+CD25+ T cells. *Immunity.* 2003;19:877–889.
48. Pasare C, Medzhitov R. Toll pathway-dependent blockade of CD4+CD25+ T cell-mediated suppression by dendritic cells. *Science.* 2003;299:1033–1036.
49. Conant SB, Swanborg RH. Autoreactive T cells persist in rats protected against experimental autoimmune encephalomyelitis and can be activated through stimulation of innate immunity. *J Immunol.* 2004;172:5322–5328.
50. Yang Y, Huang CT, Huang X, et al. Persistent Toll-like receptor signals are required for reversal of regulatory T cell-mediated CD8 tolerance. *Nat Immunol.* 2004;5:508–515.
51. Ioannou Y, Isenberg DA. Current evidence for the induction of autoimmune rheumatic manifestations by cytokine therapy. *Arthritis Rheum.* 2000;43:1431–1442.
52. Blanco P, Palucka AK, Gill M, et al. Induction of dendritic cell differentiation by IFN-alpha in systemic lupus erythematosus. *Science.* 2001;294:1540–1543.
53. Rounblom L, Alm GV. A pivotal role for the natural interferon alpha-producing cells (plasmacytoid dendritic cells) in the pathogenesis of lupus. *J Exp Med.* 2001;194:F59–F63.
54. Baechler EC, Batliwalla FM, Karypis G, et al. Interferon-inducible gene expression signature in peripheral blood cells of patients with severe lupus. *Proc Natl Acad Sci USA.* 2003;100:2610–2615.
55. Crow MK. Interferon-alpha: a new target for therapy in systemic lupus erythematosus? *Arthritis Rheum.* 2003;48:2396–2401.
56. Leadbetter EA, Rifkin IR, Hohlbaum AM, et al. Chromatin-IgG complexes activate B cells by dual engagement of IgM and Toll-like receptors. *Nature.* 2002;416:603–607.
57. Viglianti GA, Lau CM, Hanley TM, et al. Activation of autoreactive B cells by CpG dsDNA. *Immunity.* 2003;19:837–847.
58. Klinman DM. Immunotherapeutic uses of CpG oligodeoxynucleotides. *Nat Rev Immunol.* 2004;4:249–259.
59. Geary R, Yu R, Leeds J, et al. Pharmacokinetic properties in animals. In: Crooke ST, ed. *Antisense Drug Technology Principles, Strategies, and Applications.* New York: Marcel Dekker, Inc, 2001:119–154.
60. Jung J, Yi AK, Zhang X, et al. Distinct response of human B cell subpopulations in recognition of an innate immune signal, CpG DNA. *J Immunol.* 2002;169:2368–2373.
61. Verthelyi D, Ishii KJ, Gursel M, et al. Human peripheral blood cells differentially recognize and respond to two distinct CpG motifs. *J Immunol.* 2001;166:2372–2377.
62. Bernasconi NL, Traggiai E, Lanzavecchia A. Maintenance of serological memory by polyclonal activation of human memory B cells. *Science.* 2002;298:2199–2202.
63. Bernasconi NL, Onai N, Lanzavecchia A. A role for Toll-like receptors in acquired immunity: up-regulation of TLR9 by BCR triggering in naive B cells and constitutive expression in memory B cells. *Blood.* 2003; 101:4500 4504.
64. Vollmer J, Weeratna R, Payette P, et al. Characterization of three CpG oligodeoxynucleotide classes with distinct immunostimulatory activities. *Eur J Immunol.* 2004;34:251–262.
65. Marshall JD, Fearon K, Abbate C, et al. Identification of a novel CpG DNA class and motif that optimally stimulate B cell and plasmacytoid dendritic cell functions. *J Leukoc Biol.* 2003;73:781–792.
66. Hartmann G, Battiany J, Poeck H, et al. Rational design of new CpG oligonucleotides that combine B cell activation with high IFN-alpha induction in plasmacytoid dendritic cells. *Eur J Immunol.* 2003;33:1633–1641.
67. Weiner GJ, Liu HM, Wooldridge JE, et al. Immunostimulatory oligodeoxynucleotides containing the CpG motif are effective as immune adjuvants in tumor antigen immunization. *Proc Natl Acad Sci USA.* 1997;94:10833–10837.
68. Lipford GB, Bauer M, Blank C, et al. CpG-containing synthetic oligonucleotides promote B and cytotoxic T cell responses to protein antigen: a new class of vaccine adjuvants. *Eur J Immunol.* 1997;27:2340–2344.
69. Chu RS, Targoni OS, Krieg AM, et al. CpG oligodeoxynucleotides act as adjuvants that switch on T helper 1 (Th1) immunity. *J Exp Med.* 1997; 186:1623–1631.
70. Davis HL, Weeratna R, Waldschmidt TJ, et al. CpG DNA is a potent enhancer of specific immunity in mice immunized with recombinant hepatitis B surface antigen. [published erratum appears in J Immunol 1999 Mar 1;162(5):3103] *J Immunol.* 1998;160:870–876.
71. Roman M, Martin-Orozco E, Goodman JS, et al. Immunostimulatory DNA sequences function as T helper-1-promoting adjuvants. *Nat Med.* 1997;3:849–854.
72. Jones TR, Obaldia N III, Gramzinski RA, et al. Synthetic oligodeoxynucleotides containing CpG motifs enhance immunogenicity of a peptide malaria vaccine in *Aotus* monkeys. *Vaccine.* 1999;17:3065–3071.
73. Davis HL, Suparto II, Weeratna RR, et al. CpG DNA overcomes hyporesponsiveness to hepatitis B vaccine in orangutans. *Vaccine.* 2000; 18:1920–1924.
74. Halperin SA, Van Nest G, Smith B, et al. A phase I study of the safety and immunogenicity of recombinant hepatitis B surface antigen co-administered with an immunostimulatory phosphorothioate oligonucleotide adjuvant. *Vaccine.* 2003;21:2461–2467.